PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6: WO 98/08540 (11) International Publication Number: A1 A61K 39/00, 39/38, 38/08, C12P 21/06, (43) International Publication Date: 5 March 1998 (05.03.98) 21/04, 21/08, C12N 15/00, 15/09, 15/63, 15/70, 15/74, C07K 16/00 (81) Designated States: AU, CA, JP, European patent (AT, BE, (21) International Application Number: PCT/US97/15394 CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). (22) International Filing Date: 28 August 1997 (28.08.97) Published (30) Priority Data: 08/704,159 28 August 1996 (28.08.96) US With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of OPHIDIAN PHARMACEUTICALS, INC. amendments. (71) Applicant: [US/US]; 5445 East Cheryl Parkway, Madison, WI 53711 (72) Inventors: WILLIAMS, James, A.; 6420 Pueblo Court, Lincoln, NE 68516 (US). THALLEY, Bruce, S.; 126 Marinette Trail, Madison, WI 53705 (US). (74) Agents: CARROLL, Peter, G. et al.; Medlen & Carroll, LLP, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US). (54) Title: MULTIVALENT VACCINE FOR CLOSTRIDIUM BOTULINUM NEUROTOXIN

(57) Abstract

The present invention includes recombinant proteins derived from Clostridium botulinum toxins. In particular, soluble recombinant Clostridium botulinum type A, type B and type E toxin proteins are provided. Methods which allow for the isolation of recombinant proteins free of significant endotoxin contamination are provided. The soluble, endotoxin-free recombinant proteins are used as immunogens for the production of vaccines and antitoxins. These vaccines and antitoxins are useful in the treatment of humans and other animals at risk of intoxication with clostridial toxin.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	. Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
ΑÜ	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG .	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Treland	MN	Mongolia	UA	Ukraine
BR	Brazil	II.	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	iceland	MW	Malawi	US	United States of America
CA	Canada	IT.	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	P1.	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ.	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	1.3	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

MULTIVALENT VACCINE FOR CLOSTRIDIUM BOTULINUM NEUROTOXIN

FIELD OF THE INVENTION

The present invention relates to the isolation of polypeptides derived from *Clostridium* botulinum neurotoxins and the use thereof as immunogens for the production of vaccines. including multivalent vaccines, and antitoxins.

BACKGROUND OF THE INVENTION

The genus Clostridium is comprised of gram-positive, anaerobic, spore-forming bacilli. The natural habitat of these organisms is the environment and the intestinal tracts of humans and other animals. Indeed, clostridia are ubiquitous: they are commonly found in soil, dust, sewage, marine sediments, decaying vegetation, and mud. [See e.g., P.H.A. Sneath et al., "Clostridium," Bergey's Manual R of Systematic Bacteriology, Vol. 2, pp. 1141-1200.

Williams & Wilkins (1986).] Despite the identification of approximately 100 species of Clostridium, only a small number have been recognized as etiologic agents of medical and veterinary importance. Nonetheless, these species are associated with very serious diseases, including botulism, tetanus, anaerobic cellulitis, gas gangrene, bacteremia, pseudomembranous colitis, and clostridial gastroenteritis. Table 1 lists some of the species of medical and veterinary importance and the diseases with which they are associated. As virtually all of these species have been isolated from fecal samples of apparently healthy persons, some of these isolates may be transient, rather than permanent residents of the colonic flora.

TABLE 1

Clostridium Species Of Medical And Veterinary Importance*

Species	Disease	
C. aminovalericum	Bacteriuria (pregnant women)	
C argentinense	Infected wounds: Bacteremia: Botulism: Infections of amniotic fluid	
C. baratii	Infected war wounds: Peritonitis: Infectious processes of the eye, ear and prostate	
C. heijerinekikii	Infected wounds	
C. bifermentans	Infected wounds: Abscesses: Gas Gangrene: Bacteremia	
C. boudinum	Food poisoning: Botulism (wound, food, infant)	
C. butyricum	Urinary tract, lower respiratory tract, pleural cavity, and abdominal infections; Infected wounds; Abscesses: Bacteremia	
C. cadaveris	Abscesses: Infected wounds	

- 30

5

10

15

20

25

5

10

15

20

25

TABLE 1

Clostridium Species Of Medical And Veterinary Importance* Species				
	Disease			
C. carnis	Soft tissue infections: Bacteremia			
C. chanvoci	Blackleg			
C. clostridioforme	Abdominal, cervical, scrotal, pleural, and other infections: Septicemia: Peritonitis; Appendicitis			
C. cochlearnm	Isolated from human disease processes, but role in disease unknown.			
C. difficile	Antimicrobial-associated diarrhea: Pseudomembranous enterocolitis: Bacteremia: Pyogenic infections			
C. fallax	Soft tissue infections			
C. ghnoii	Soft tissue infections			
C. glycolicim	Wound infections: Abscesses: Peritonitis			
C. hastiforme	Infected war wounds: Bacteremia: Abscesses			
C histolyticum	Infected war wounds: Gas gangrene: Gingival plaque isolate			
C. mdolis	Gastrointestinal tract infections			
C innocuum	Gastrointestinal tract infections: Empyema			
C nregulare	Penile lesions			
C. leptum	Isolated from human disease processes, but role in disease unknown.			
C limosum	Bacteremia: Peritonitis: Pulmonary infections			
C. malenominatum	Various infectious processes			
С. поуу	Infected wounds: Gas gangrene: Blackleg. Big head (ovine): Redwater disease (bovine)			
C. oroticum	Urinary tract infections: Rectal abscesses			
C. paraputrificum	Bacteremia: Peritonitis: Infected wounds: Appendicitis			
C. perfringens	Gas gangrene: Anaerobic cellulitis: Intra-abdominal abscesses: Soft tissue infections: Food poisoning: Necrotizing pneumonia: Empyema: Meningitis: Bacteremia: Uterine Infections; Enteritis necrotans: Lamb dysentery: Struck: Ovine Enterotoxemia:			
) putrefaciens	Bacteriuria (Pregnant women with bacteremia)			
. putrificum	Abscesses: Infected wounds: Bacteremia			
C. romosum	Infections of the abdominal cavity, genital tract, lung, and biliary tract; Bacteremia			
surtagoforme	Isolated from human disease processes, but role in disease unknown.			
'. septicum	Gas gangrene: Bacteremia: Suppurative infections: Necrotizing enterocolitis: Braxy			
`. sordellii	Gas gangrene: Wound infections: Penile lesions: Bacteremia: Abscesses: Abdominal and vaginal infections			

5

10

15

20

25

TABLE !
Clostridium Species Of Medical And Veterinary Importance*

	· · · · · · · · · · · · · · · · · · ·		
Species	Appendicitis: Bacteremia: Bone and soft tissue infections: Intraperitoneal infections: Infected war wounds: Visceral gas gangrene Renal abscesses		
C. sphenoides			
C. sporogenes	Gas gangrene: Bacteremia: Endocarditis: central nervous system and pleuropulmonary infections: Penile lesions: Infected war wounds: Other pyogenic infections		
C. subterminale	Bacteremia: Empyema: Biliary tract, soft tissue and bone infections		
C symbiosum	Liver abscesses: Bacteremia: Infections resulting due to bowel flora		
C. tertum	Gas gangrene: Appendicitis: Brain abscesses: Intestinal tract and soft tissue infections: Infected war wounds: Periodontitis: Bacteremia		
C. tetani	Tetanus: Infected gums and teeth: Corneal ulcerations: Mastoid and middle ear infections: Intraperitoneal infections: Tetanus neonatorum: Postpartum uterine infections: Soft tissue infections, especially related to trauma (including abrasions and lacerations): Infections related to use of contaminated needles		
C. thermosaccharolyticum	Isolated from human disease processes, but role in disease unknown.		

Compiled from P.G. Engelkirk et al. "Classification", Principles and Practice of Clinical Anaerohic Bacteriology, pp. 22-23. Star Publishing Co., Belmont, CA (1992); J. Stephen and R.A. Petrowski, "Toxins Which Traverse Membranes and Deregulate Cells," in Bacterial Toxins, 2d ed., pp. 66-67. American Society for Microbiology (1986); R. Berkow and A.J. Fletcher (eds.), "Bacterial Diseases," Merck Manual of Diagnosis and Therapy, 16th ed., pp. 116-126, Merck Research Laboratories, Rahway, N.J. (1992); and O.H. Sigmund and C.M. Fraser (eds.), "Clostridial Infections," Merck Veterinary Manual, 5th ed., pp. 396-409, Merck & Co., Rahway, N.J. (1979).

In most cases, the pathogenicity of these organisms is related to the release of powerful exotoxins or highly destructive enzymes. Indeed, several species of the genus *Clostridium* produce toxins and other enzymes of great medical and veterinary significance. [C.L. Hatheway, Clin. Microbiol. Rev. 3:66-98 (1990).]

Perhaps because of their significance for human and veterinary medicine, much research has been conducted on these toxins, in particular those of *C. hotulinum* and *C. difficile*.

C. botulinum

Several strains of *Clostridium botulinum* produce toxins of significance to human and animal health. [C.L. Hatheway, Clin. Microbiol. Rev. 3:66-98 (1990)]. The effects of these toxins range from diarrheal diseases that can cause destruction of the colon, to paralytic effects that can cause death. Particularly at risk for developing clostridial diseases are

neonates and humans and animals in poor health (e.g., those suffering from diseases associated with old age or immunodeficiency diseases).

5

10

15

20

25

30

Clostridium botulinum produces the most poisonous biological toxin known. The lethal human dose is a mere 10° mg/kg bodyweight for toxin in the bloodstream. Botulinal toxin blocks nerve transmission to the muscles, resulting in flaccid paralysis. When the toxin reaches airway and respiratory muscles, it results in respiratory failure that can cause death. [S. Arnon, J. Infect. Dis. 154:201-206 (1986)]

C. botulinum spores are carried by dust and are found on vegetables taken from the soil, on fresh fruits, and on agricultural products such as honey. Under conditions favorable to the organism, the spores germinate to vegetative cells which produces toxin. [S. Arnon, Ann. Rev. Med. 31:541 (1980)]

Botulism disease may be grouped into four types, based on the method of introduction of toxin into the bloodstream. Food-borne botulism results from ingesting improperly preserved and inadequately heated food that contains botulinal toxin. There were 355 cases of food-borne botulism in the United States between 1976 and 1984. [K.L. MacDonald et al., Am. J. Epidemiol. 124:794 (1986).] The death rate due to botulinal toxin is 12% and can be higher in particular risk groups. [C.O. Tacket et al., Am. J. Med. 76:794 (1984).] Woundinduced botulism results from C. botulinum penetrating traumatized tissue and producing toxin that is absorbed into the bloodstream. Since 1950, thirty cases of wound botulism have been reported. [M.N. Swartz, "Anaerobic Spore-Forming Bacilli: The Clostridia," pp. 633-646, in B.D. Davis et al. (eds.). Microbiology, 4th edition, J.B. Lippincott Co. (1990). [Inhalation botulism results when the toxin is inhaled. Inhalation botulism has been reported as the result of accidental exposure in the laboratory [E. Holzer, Med. Klin, 41:1735 (1962)] and could arise if the toxin is used as an agent of biological warfare [D.R. Franz et al., in Botulinum and Tetanus Neurotoxins, B.R. DasGupta, ed., Plenum Press, New York (1993), pp. 473-4761. Infectious infant botulism results from C. botulinum colonization of the infant intestine with production of toxin and its absorption into the bloodstream. It is likely that the bacterium gains entry when spores are ingested and subsequently germinate. [S. Arnon, J. Infect. Dis. 154:201 (1986).] There have been 500 cases reported since it was first recognized in 1976. [M.N. Swartz, supra.]

Infant botulism strikes infants who are three weeks to eleven months old (greater than 90% of the cases are infants less than six months). [S. Arnon, J. Infect. Dis. 154:201 (1986).] It is believed that infants are susceptible, due, in large part, to the absence of the full adult complement of intestinal microflora. The benign microflora present in the adult intestine provide an acidic environment that is not favorable to colonization by *C. botulinum*. Infants begin life with a sterile intestine which is gradually colonized by microflora. Because of the limited microflora present in early infancy, the intestinal environment is not as acidic, allowing for *C. botulinum* spore germination, growth, and toxin production. In this regard, some adults who have undergone antibiotic therapy which alters intestinal microflora become more susceptible to botulism.

5

10

15

20

25

30

An additional factor accounting for infant susceptibility to infectious botulism is the immaturity of the infant immune system. The mature immune system is sensitized to bacterial antigens and produces protective antibodies. Secretory IgA produced in the adult intestine has the ability to agglutinate vegetative cells of *C. botulinum*. [S. Arnon, J. Infect. Dis. 154:201 (1986).] Secretory IgA may also act by preventing intestinal bacteria and their products from crossing the cells of the intestine. [S. Arnon, Epidemiol, Rev. 3:45 (1981).] The infant immune system is not primed to do this.

Clinical symptoms of infant botulism range from mild paralysis, to moderate and severe paralysis requiring hospitalization, to fulminant paralysis, leading to sudden death. [S. Arnon, Epidemiol, Rev. 3:45 (1981).]

The chief therapy for severe infant botulism is ventilatory assistance using a mechanical respirator and concurrent elimination of toxin and bacteria using cathartics, enemas, and gastric lavage. There were 68 hospitalizations in California for infant botulism in a single year with a total cost of over \$4 million for treatment. [T.L. Frankovich and S. Arnon. West. J. Med. 154:103 (1991).]

Different strains of Clostridium botulinum each produce antigenically distinct toxin designated by the letters A-G. Serotype A toxin has been implicated in 26% of the cases of food botulism: types B, E and F have also been implicated in a smaller percentage of the food botulism cases [II. Sugiyama, Microbiol, Rev. 44:419 (1980)]. Wound botulism has been reportedly caused by only types A or B toxins [H. Sugiyama, supra]. Nearly all cases of infant botulism have been caused by bacteria producing either type A or type B toxin.

(Exceptionally, one New Mexico case was caused by Clostridium botulinum producing type F toxin and another by Clostridium botulinum producing a type B-type F hybrid.) [S. Arnon, Epidemiol. Rev. 3:45 (1981).] Type C toxin affects waterfowl, cattle, horses and mink. Type D toxin affects cattle, and type E toxin affects both humans and birds.

5

A trivalent antitoxin derived from horse plasma is commercially available from Connaught Industries Ltd. as a therapy for toxin types A. B. and E. However, the antitoxin has several disadvantages. First, extremely large dosages must be injected intravenously and/or intramuscularly. Second, the antitoxin has serious side effects such as acute anaphylaxis which can lead to death, and serum sickness. Finally, the efficacy of the antitoxin is uncertain and the treatment is costly. [C.O. Tacket et al., Am. J. Med. 76:794 (1984).]

10

A heptavalent equine botulinal antitoxin which uses only the F(ab')2 portion of the antibody molecule has been tested by the United States Military. [M. Balady, USAMRDC Newsletter, p. 6 (1991).] This was raised against impure toxoids in those large animals and is not a high titer preparation.

15

A pentavalent human antitoxin has been collected from immunized human subjects for use as a treatment for infant botulism. The supply of this antitoxin is limited and cannot be expected to meet the needs of all individuals stricken with botulism disease. In addition, collection of human sera must involve screening out HIV and other potentially serious human pathogens. [P.J. Schwarz and S.S. Arnon, Western J. Med. 156:197 (1992).]

20

Infant botulism has been implicated as the cause of mortality in some cases of Sudden Infant Death Syndrome (SIDS, also known as crib death). SIDS is officially recognized as infant death that is sudden and unexpected and that remained unexplained despite complete post-mortem examination. The link of SIDS to infant botulism came when fecal or blood specimens taken at autopsy from SIDS infants were found to contain *C. botulinum* organisms and/or toxin in 3-4% of cases analyzed. [D.R. Peterson *et al.*, Rev. Infect. Dis. 1:630 (1979).] In contrast, only 1 of 160 healthy infants (0.6%) had *C. botulinum* organisms in the feces and no botulinal toxin. (S. Arnon *et al.*, Lancet, pp. 1273-76, June 17, 1978.)

30

25

In developed countries, SIDS is the number one cause of death in children between one month and one year old. (S. Arnon *et al.*, Lancet, pp. 1273-77, June 17, 1978.) More children die from SIDS in the first year than from any other single cause of death in the first

fourteen years of life. In the United States, there are 8.000-10.000 SIDS victims annually. *Id.*

What is needed is an effective therapy against infant botulism that is free of dangerous side effects, is available in large supply at a reasonable price, and can be safely and gently delivered so that prophylactic application to infants is feasible.

Immunization of subjects with toxin preparations has been done in an attempt to induce immunity against botulinal toxins. A C. botulinum vaccine comprising chemically inactivated (i.e., formaldehyde-treated) type A, B, C, D and E toxin is commercially available for human usage. However, this vaccine preparation has several disadvantages. First, the efficacy of this vaccine is variable (in particular, only 78% of recipients produce protective levels of anti-type B antibodies following administration of the primary series). Second, immunization is painful (deep subcutaneous inoculation is required for administration), with adverse reactions being common (moderate to severe local reactions occur in approximately 6% of recipients upon initial injection; this number rises to approximately 11% of individuals who receive booster injections) [Informational Brochure for the Pentavalent (ABCDE) Botulinum Toxoid, Centers for Disease Control]. Third, preparation of the vaccine is dangerous as active toxin must be handled by laboratory workers.

What is needed are safe and effective vaccine preparations for administration to those at risk of exposure to C. botulinum toxins.

C. difficile

. 5

10

15

20

25

30

C. difficile, an organism which gained its name due to difficulties encountered in its isolation, has recently been proven to be an etiologic agent of diarrheal disease. (Sneath et al., p. 1165.). C. difficile is present in the gastrointestinal tract of approximately 3% of healthy adults, and 10-30% of neonates without adverse effect (Swartz, at p. 644); by other estimates. C. difficile is a part of the normal gastrointestinal flora of 2-10% of humans. [G.F. Brooks et al., (eds.) "Infections Caused by Anaerobic Bacteria," Jawetz, Melnick, & Adelberg's Medical Microbiology, 19th ed., pp. 257-262, Appleton & Lange, San Mateo, CA (1991).] As these organisms are relatively resistant to most commonly used antimicrobials, when a patient is treated with antibiotics, the other members of the normal gastrointestinal

flora are suppressed and *C. difficile* flourishes, producing cytopathic toxins and enterotoxins. It has been found in 25% of cases of moderate diarrhea resulting from treatment with antibiotics, especially the cephalosporins, clindamycin, and ampicillin. [M.N. Swartz at 644.]

Importantly, C. difficile is commonly associated with nosocomial infections. The organism is often present in the hospital and nursing home environments and may be carried on the hands and clothing of hospital personnel who care for debilitated and immunocompromised patients. As many of these patients are being treated with antimicrobials or other chemotherapeutic agents, such transmission of C. difficile represents a significant risk factor for disease. (Engelkirk et al., pp. 64-67.)

10

15

5

C. difficile is associated with a range of diarrhetic illness, ranging from diarrhea alone to marked diarrhea and necrosis of the gastrointestinal mucosa with the accumulation of inflammatory cells and fibrin, which forms a pseudomembrane in the affected area. (Brooks et al.) It has been found in over 95% of pseudomembranous enterocolitis cases. (Swartz, at p. 644.) This occasionally fatal disease is characterized by diarrhea, multiple small colonic plaques, and toxic megacolon. (Swartz, at p. 644.) Although stool cultures are sometimes used for diagnosis, diagnosis is best made by detection of the heat labile toxins present in fecal filtrates from patients with enterocolitis due to C. difficile. (Swartz, at p. 644-645; and Brooks et al., at p. 260.) C. difficile toxins are cytotoxic for tissue/cell cultures and cause enterocolitis when injected intracecally into hamsters. (Swartz, at p. 644.)

20

25

The enterotoxicity of *C. difficile* is primarily due to the action of two toxins. designated A and B, each of approximately 300,000 in molecular weight. Both are potent cytotoxins, with toxin A possessing direct enterocytotoxic activity. [Lyerly *et al.*, Infect. Immun. 60:4633 (1992).] Unlike toxin A of *C. perfringens*, an organism rarely associated with antimicrobial-associated diarrhea, the toxin of *C. difficile* is not a spore coat constituent and is not produced during sporulation. (Swartz, at p. 644.) *C. difficile* toxin A causes hemorrhage, fluid accumulation and mucosal damage in rabbit ileal loops and appears to increase the uptake of toxin B by the intestinal mucosa. Toxin B does not cause intestinal fluid accumulation, but it is 1000 times more toxic than toxin A to tissue culture cells and causes membrane damage. Although both toxins induce similar cellular effects such as actin disaggregation, differences in cell specificity occurs.

30

Both toxins are important in disease. [Borriello et al., Rev. Infect. Dis., 12(suppl. 2):S185 (1990): Lyerly et al., Infect. Immun., 47:349 (1985): and Rolfe. Infect. Immun., 59:1223 (1990).] Toxin A is thought to act first by binding to brush border receptors, destroying the outer mucosal layer, then allowing toxin B to gain access to the underlying tissue. These steps in pathogenesis would indicate that the production of neutralizing antibodies against toxin A may be sufficient in the prophylactic therapy of CDAD. However, antibodies against toxin B may be a necessary additional component for an effective therapeutic against later stage colonic disease. Indeed, it has been reported that animals require antibodies to both toxin A and toxin B to be completely protected against the disease. [Kim and Rolfe, Abstr. Ann. Meet. Am. Soc. Microbiol., 69:62 (1987).]

. 5 .

10

15

20

25

C. difficile has also been reported to produce other toxins such as an enterotoxin different from toxins A and B [Banno et al., Rev. Infect. Dis., 6(Suppl. 1:S11-S20 (1984)], a low molecular weight toxin [Rihn et al., Biochem. Biophys. Res. Comm., 124:690-695 (1984)], a motility altering factor [Justus et al., Gastroenterol., 83:836-843 (1982)], and perhaps other toxins. Regardless. C. difficile gastrointestinal disease is of primary concern.

It is significant that due to its resistance to most commonly used antimicrobials. C. difficile is associated with antimicrobial therapy with virtually all antimicrobial agents (although most commonly ampicillin, clindamycin and cephalosporins). It is also associated with disease in patients undergoing chemotherapy with such compounds as methotrexate. 5-fluorouracil, cyclophosphamide, and doxorubicin. [S.M. Finegold et al., Clinical Guide to Anaerobic Infections, pp. 88-89. Star Publishing Co., Belmont, CA (1992).]

Treatment of *C. difficile* disease is problematic, given the high resistance of the organism. Oral metronidazole, bacitracin and vancomycin have been reported to be effective. (Finegold *et al.*, p. 89.) However there are problems associated with treatment utilizing these compounds. Vancomycin is very expensive, some patients are unable to take oral medication, and the relapse rate is high (20-25%), although it may not occur for several weeks. *Id.*

C. difficile disease would be prevented or treated by neutralizing the effects of these toxins in the gastrointestinal tract. Thus, what is needed is an effective therapy against C. difficile toxin that is free of dangerous side effects, is available in large supply at a reasonable

price, and can be safely delivered so that prophylactic application to patients at risk of developing pseudomembranous enterocolitis can be effectively treated.

DESCRIPTION OF THE DRAWINGS

5

- Figure 1 shows the reactivity of anti-C. hotulinum IgY by Western blot.
- Figure 2 shows the IgY antibody titer to C. *botulinum* type A toxoid in eggs, measured by ELISA.
 - Figure 3 shows the results of C. difficile toxin A neutralization assays.
 - Figure 4 shows the results of C. difficile toxin B neutralization assays.
- Figure 5 shows the results of C. difficile toxin B neutralization assays.
 - Figure 6 is a restriction map of C. difficile toxin A gene, showing sequences of primers 1-4 (SEQ ID NOS:1-4).
 - Figure 7 is a Western blot of C. difficile toxin A reactive protein.
 - Figure 8 shows C. difficile toxin A expression constructs.
- Figure 9 shows C difficile toxin Λ expression constructs.
 - Figure 10 shows the purification of recombinant C. difficile toxin A.
 - Figure 11 shows the results of *C. difficile* toxin A neutralization assays with antibodies reactive to recombinant toxin A.
 - Figure 12 shows the results for a C. difficile toxin A neutralization plate.
- 20 Figure 13 shows the results for a C. difficile toxin A neutralization plate.
 - Figure 14 shows the results of recombinant C. difficile toxin A neutralization assays.
 - Figure 15 shows C. difficile toxin A expression constructs.
 - Figure 16 shows a chromatograph plotting absorbance at 280 nm against retention time for a pMA1870-680 IgY PEG preparation.
- 25 Figure 17 shows two recombinant C. difficile toxin B expression constructs.
 - Figure 18 shows C. difficile toxin B expression constructs.
 - Figure 19 shows C. difficile toxin B expression constructs.
 - Figure 20 shows C. difficile toxin B expression constructs.
- Figure 21 is an SDS-PAGE gel showing the purification of recombinant *C. difficile* toxin B fusion protein.

Figure 22 is an SDS-PAGE gel showing the purification of two histidine-tagged recombinant C. difficile toxin B proteins.

Figure 23 shows C. difficile toxin B expression constructs.

. 5

10

15

20

25

30

- Figure 24 is a Western blot of C. difficile toxin B reactive protein.
- Figure 25 shows C. botulinum type A toxin expression constructs; constructs used to provide C. botulinum or C. difficile sequences are also shown.
- Figure 26 is an SDS-PAGE gel stained with Coomaisse blue showing the purification of recombinant *C. botulinum* type A toxin fusion proteins.
- Figure 27 shows C. botulinum type A toxin expression constructs: constructs used to provide C. botulinum sequences are also shown.
- Figure 28 is an SDS-PAGE gel stained with Coomaisse blue showing the purification of pHisBot protein using the Ni-NTA resin.
- Figure 29 is an SDS-PAGE gel stained with Coomaisse blue showing the expression of pHisBot protein in BL21(DE3) and BL21(DE3)pLysS host cells.
- Figure 30 is an SDS-PAGE gel stained with Coomaisse blue showing the purification of pHisBot protein using a batch absorption procedure.
- Figure 31 is an SDS-PAGE gel stained with Coomaisse blue showing the purification of pHisBot and pHisBot(native) proteins using a Ni-NTA column.
- Figure 32 is an SDS-PAGE gel stained with Coomaisse blue showing the purification of pHisBotA protein expressed in pHisBotA(syn) kan laclq T7/pACYCGro/BL21(DE3) cells using an IDA column.
- Figure 33 is an SDS-PAGE gel stained with Coomaisse blue showing the purification of pHisBotA, pHisBotB and pHisBotE proteins by IDA chromatography followed by chromatography on S-100 to remove folding chaperones.
- Figure 34 is an SDS-PAGE gel stained with Coomaisse blue showing the extracts derived from pHisBotB amp T7lac/BL21(DE3) cells before and after purification on a Ni-NTA column.
- Figure 35 is an SDS-PAGE gel run under native conditions and stained with Coomaisse blue showing the removal of folding chaperones from IDA-purified BotB protein using a S-100 column.

Figure 36 is an SDS-PAGE gel stained with Coomaisse blue showing proteins that eluted during an imidazole step gradient applied to a IDA column containing a lysate of pHisBotB kan laclq T7/pACYCGro/BL21(DE3) cells.

Figure 37 is an SDS-PAGE gel run under native conditions and stained with Coomaisse blue showing IDA-purified BotB protein before and after ultrafiltration.

Figure 38 is an SDS-PAGE gel stained with Coomaisse blue showing the purification of BotE protein using a NiNTA column.

Figure 39 is an SDS-PAGE gel stained with Coomaisse blue showing extracts derived from pHisBotA kan T7 lac/BL21(DE3) pLysS cells grown in fermentation culture.

Figure 40 is a chromatogram showing proteins present after IDA-purified BotE protein was applied to a S-100 column.

DEFINITIONS

5 .

10

15

20

25

30

To facilitate understanding of the invention, a number of terms are defined below.

As used herein, the term "neutralizing" is used in reference to antitoxins, particularly antitoxins comprising antibodies, which have the ability to prevent the pathological actions of the toxin against which the antitoxin is directed.

As used herein, the term "overproducing" is used in reference to the production of clostridial toxin polypeptides in a host cell and indicates that the host cell is producing more of the clostridial toxin by virtue of the introduction of nucleic acid sequences encoding said clostridial toxin polypeptide than would be expressed by said host cell absent the introduction of said nucleic acid sequences. To allow ease of purification of toxin polypeptides produced in a host cell it is preferred that the host cell express or overproduce said toxin polypeptide at a level greater than 1 mg/liter of host cell culture.

"A host cell capable of expressing a recombinant protein at a level greater than or equal to 5% of the total cellular protein" is a host cell in which the recombinant protein represents at least 5% of the total cellular protein. To determine what percentage of total cellular protein the recombinant protein represents, the following steps are taken. A total of 10 OD₆₀₀ units of recombinant host cells (e.g., 200 µl of cells at OD₆₀₀ 50/ml) are removed (at a timepoint known to represent the peak of expression of the desired recombinant protein) to a 1.5 ml microfuge tube and pelleted for 2 min at maximum rpm in a microfuge. The

pellets are resuspended in 1 ml of 50 mM NaHPO4, 0.5 M NaCl, 40 mM imidazole buffer (pH 6.8) containing 1 mg/ml lysozyme. The samples are incubated for 20 min at room temperature and stored ON at -70°C. Samples are thawed completely at room temperature and sonicated 2 X 10 seconds with a Branson Sonifier 450 microtip probe at # 3 power setting. The samples are centrifuged for 5 min. at maximum rpm in a microfuge. An aliquot (20 μ l) of the protein sample is removed to 20 μ l 2X sample buffer (this represents the total protein extract). The samples are heated to 95°C for 5 min, then cooled and 5 or 10 µl are loaded onto 12.5% SDS-PAGE gels. High molecular weight protein markers are also loaded to allow for estimation of the MW of identified recombinant proteins. After electrophoresis, protein is detected generally by staining with Coomassie blue and the stained gel is scanned using a densitometer to determine the percentage of protein present in each band. In this , manner, the percentage of protein present in the band corresponding to the recombinant protein of interest may be determined. It is not necessary that Coomassie blue be employed for the detection of protein, a number of fluorescent dyes [e.g., Sypro orange S-6651 (Molecular Probes, Eugene, OR) may be employed and the stained gel scanned using a fluoroimager [e.g., Fluor Imager SI (Molecular Dynamics, Sunnyvale, CA)].

- 5

10

15

20

25

30

"A host cell capable of expressing a recombinant protein as a soluble protein at a level greater than or equal to 0.25% of the total soluble cellular protein" is a host cell in which the amount of soluble recombinant protein present represents at least 0.25% of the total cellular protein. As used herein "total soluble cellular protein" refers to a clarified PEI lysate prepared as described in Example 31(c)(iv). Briefly, cells are harvested following induction of expression of recombinant protein (at a point of maximal expression). The cells are resuspended in cell resuspension buffer (CRB: 50 mM NaPO₄, 0.5 M NaCl. 40 mM imidazole, pH 6.8) to create a 20% cell suspension (wet weight of cells/volume of CRB) and cell lysates are prepared as described in Example 31(c)(iv) (i.e., sonication or homogenization followed by centrifugation). The cell lysate is then flocculated utilizing polyethylencimine (PEI) prior to centrifugation. PEI (a 2% solution in dH₂O, pH 7.5 with HCl) is added to the cell lysate to a final concentration of 0.2%, and stirred for 20 min at room temperature prior to centrifugation [8.500 rpm in JA10 rotor (Beckman) for 30 minutes at 4°C]. This treatment removes RNA, DNA and cell wall components, resulting in a clarified, low viscosity lysate ("PEI clarified lysate"). The recombinant protein present in the PEI clarified lysate is then

purified (e.g., by chromatography on an IDA column for his-tagged proteins). The amount of purified recombinant protein (i.e., the eluted protein) is divided by the concentration of protein present in the PEI clarified lysate (typically 8 mg/ml when using a 20% cell suspension as the starting material) and multiplied by 100 to determine what percentage of total soluble cellular protein is comprised of the soluble recombinant protein (see Example 33b).

5

10

15

20

25

30

As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (i.e., C. botulinum toxin A, B, C, D, E, F, or G and fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-toxin protein). The fusion partner may enhance solubility of the C. botulinum protein as expressed in a host cell, may provide an affinity tag to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both. If desired, the fusion protein may be removed from the protein of interest (i.e., toxin protein or fragments thereof) prior to immunization by a variety of enzymatic or chemical means known to the art.

As used herein the term "non-toxin protein" or "non-toxin protein sequence" refers to that portion of a fusion protein which comprises a protein or protein sequence which is not derived from a bacterial toxin protein.

The term "protein of interest" as used herein refers to the protein whose expression is desired within the fusion protein. In a fusion protein the protein of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

As used herein, the term "maltose binding protein" refers to the maltose binding protein of E, coli. A portion of the maltose binding protein may be added to a protein of interest to generate a fusion protein; a portion of the maltose binding protein may merely enhance the solubility of the resulting fusion protein when expressed in a bacterial host. On the other hand, a portion of the maltose binding protein may allow affinity purification of the fusion protein on an amylose resin.

As used herein, the term "poly-histidine tract" when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a protein of interest. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine

residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate or IDA column.

As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antitoxins are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of immunoglobulin that does not bind toxin. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind toxin results in an increase in the percent of toxin-reactive immunoglobulins in the sample. In another example, recombinant toxin polypeptides are expressed in bacterial host cells and the toxin polypeptides are purified by the removal of host cell proteins; the percent of recombinant toxin polypeptides is thereby increased in the sample. Additionally, the recombinant toxin polypeptides are purified by the removal of host cell components such as lipopolysaccharide (e.g., endotoxin).

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

The term "native protein" as used herein refers to a protein which is isolated from a natural source as opposed to the production of a protein by recombinant means.

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell is a protein which exists in solution in the cytoplasm of the host cell; if the protein contains a signal sequence the soluble protein is exported to the periplasmic space in bacteria hosts and is secreted into the culture medium in eucaryotic cells capable of secretion or by bacterial host possessing the appropriate genes (*i.e.*, the *kil* gene). In contrast, an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called inclusion bodies) in the host cell. High level expression (*i.e.*, greater than 10-20 mg recombinant protein/liter of bacterial culture) of recombinant proteins often results in the expressed protein being found in inclusion bodies in the bacterial host cells. A soluble

30

- 5

10

15

20

25

protein is a protein which is not found in an inclusion body inside the host cell or is found both in the cytoplasm and in inclusion bodies and in this case the protein may be present at high or low levels in the cytoplasm.

5

10

15

20

25

30

A distinction is drawn between a soluble protein (*i.e.*, a protein which when expressed in a host cell is produced in a soluble form) and a "solubilized" protein. An insoluble recombinant protein found inside an inclusion body may be solubilized (*i.e.*, rendered into a soluble form) by treating purified inclusion bodies with denaturants such as guanidine hydrochloride, urea or sodium dodecyl sulfate (SDS). These denaturants must then be removed from the solubilized protein preparation to allow the recovered protein to renature (refold). Not all proteins will refold into an active conformation after solubilization in a denaturant and removal of the denaturant. Many proteins precipitate upon removal of the denaturant. SDS may be used to solubilize inclusion bodies and will maintain the proteins in solution at low concentration. However, dialysis will not always remove all of the SDS (SDS can form micelles which do not dialyze out); therefore, SDS-solubilized inclusion body protein is soluble but not refolded.

A distinction is drawn between proteins which are soluble (i.e., dissolved) in a solution devoid of significant amounts of ionic detergents (e.g., SDS) or denaturants (e.g., urea, guanidine hydrochloride) and proteins which exist as a suspension of insoluble protein molecules dispersed within the solution. A soluble protein will not be removed from a solution containing the protein by centrifugation using conditions sufficient to remove bacteria present in a liquid medium (i.e., centrifugation at 12,000 x g for 4-5 minutes). For example, to test whether two proteins, protein A and protein B, are soluble in solution, the two proteins are placed into a solution selected from the group consisting of PBS-NaCl (PBS containing 0.5 M NaCl), PBS-NaCl containing 0.2% Tween 20, PBS, PBS containing 0.2% Tween 20, PBS-C (PBS containing 2 mM CaCl₂), PBS-C containing either 0.1 or 0.5 % Tween 20, PBS-C containing either 0.1 or 0.5% NP-40, PBS-C containing either 0.1 or 0.5% Triton X-100, PBS-C containing 0.1% sodium deoxycholate. The mixture containing proteins A and B is then centrifuged at 5000 x g for 5 minutes. The supernatant and pellet formed by centrifugation are then assayed for the presence of protein A and B. If protein A is found in the supernatant and not in the pellet [except for minor amounts (i.e., less than 10%) as a result of trapping], protein is said to be soluble in the solution tested. If the majority of

protein B is found in the pellet (i.e., greater than 90%), then protein B is said to exist as a suspension in the solution tested.

As used herein, the term "therapeutic amount" refers to that amount of antitoxin required to neutralize the pathologic effects of one or more clostridial toxins in a subject.

• 5

10

15

20

25

30

The term "pyrogen" as used herein refers to a fever-producing substance. Pyrogens may be endogenous to the host (e.g., prostaglandins) or may be exogenous compounds (e.g., bacterial endo- and exotoxins, nonbacterial compounds such as antigens and certain steroid compounds, etc.). The presence of pyrogen in a pharmaceutical solution may be detected using the U.S. Pharmacopeia (USP) rabbit fever test (United States Pharmacopeia, Vol. XXII (1990) United States Pharmacopeial Convention, Rockville, MD, p. 151).

The term "endotoxin" as used herein refers to the high molecular weight complexes associated with the outer membrane of gram-negative bacteria. Unpurified endotoxin contains lipids, proteins and carbohydrates. Highly purified endotoxin does not contain protein and is referred to as lipopolysaccharide (LPS). Because unpurified endotoxin is of concern in the production of pharmaceutical compounds (e.g., proteins produced in E. coli using recombinant DNA technology), the term endotoxin as used herein refers to unpurified endotoxin. Bacterial endotoxin is a well known pyrogen.

As used herein, the term "endotoxin-free" when used in reference to a composition to be administered parenterally (with the exception of intrathecal administration) to a host means that the dose to be delivered contains less than 5 EU/kg body weight [FDA Guidelines for Parenteral Drugs (December 1987)]. Assuming a weight of 70 kg for an adult human, the dose must contain less than 350 EU to meet FDA Guidelines for parenteral administration. Endotoxin levels are measured herein using the Limulus Amebocyte Lysate (LAL) test (Limulus Amebocyte Lysate Pyrochrome^{1M}, Associates of Cape Cod. Inc. Woods Hole, MA). To measure endotoxin levels in preparations of recombinant proteins, 0.5 ml of a solution comprising 0.5 mg of purified recombinant protein in 50 mM NaPO₄, pH 7.0, 0.3M NaCl and 10% glycerol is used in the LAL assay according to the manufacturer's instructions for the endpoint chromogenic without diazo-coupling method [the specific components of the buffer containing recombinant protein to be analyzed in the LAL test are not important; any buffer having a neutral pH may be employed (see for example, alternative buffers employed in Examples 34, 40 and 45)]. Compositions containing less than or equal to than 250 endotoxin

units (EU)/mg of purified recombinant protein are herein defined as "substantially endotoxin-free." Preferably the composition contains less than or equal to 100, and most preferably less than or equal to 60. (EU)/mg of purified recombinant protein. Typically, administration of bacterial toxins or toxoids to adult humans for the purpose of vaccination involves doses of about 10-500 µg protein/dose. Therefore, administration of 10-500 µg of a purified recombinant protein to a 70 kg human, wherein said purified recombinant protein preparation contains 60 EU/mg protein, results in the introduction of only 0.6 to 30 EU (i.e., 0.2 to 8.6% of the maximum allowable endotoxin burden per parenteral dose). Administration of 10-500 µg of a purified recombinant protein to a 70 kg human, wherein said purified recombinant protein preparation contains 250 EU/mg protein, results in the introduction of only 2.5 to 125 EU (i.e., 0.7 to 36% of the maximum allowable endotoxin burden per parenteral dose).

The LAL test is accepted by the U.S. FDA as a means of detecting bacterial endotoxins (21 C.F.R. §§ 660.100 -105). Studies have shown that the LAL test is equivalent or superior to the USP rabbit pyrogen test for the detection of endotoxin and thus the LAL test can be used as a surrogate for pyrogenicity studies in animals [F.C. Perason, Pyrogens: endotoxins, LAL testing and depyrogenation, Marcel Dekker, New York (1985), pp.150-155]. The FDA Bureau of Biologies accepts the LAL assay in place of the USP rabbit pyrogen test so long as the LAL assay utilized is shown to be as sensitive as, or more sensitive as the rabbit test [Fed. Reg., 38, 26130 (1980)].

The term "monovalent" when used in reference to a clostridial vaccine refers to a vaccine which is capable of provoking an immune response in a host animal directed against a single type of clostridial toxin. For example, if immunization of a host with *C. botulinum* type A toxin vaccine induces antibodies in the immunized host which protect against a challenge with type A toxin but not against challenge with type B. C. D. E. F or G toxins, then the type A vaccine is said to be monovalent. In contrast, a "multivalent" vaccine provokes an immune response in a host animal directed against several (*i.e.*, more than one) clostridial toxins. For example, if immunization of a host with a vaccine comprising *C. botulinum* type A and B toxins induces the production of antibodies which protect the host against a challenge with both type A and B toxin, the vaccine is said to be multivalent (in

particular, this hypothetical vaccine is bivalent).

5

10

15

20

25

30

As used herein the term "immunogenically-effective amount" refers to that amount of an immunogen required to invoke the production of protective levels of antibodies in a host upon vaccination.

The term "protective level", when used in reference to the level of antibodies induced upon immunization of the host with an immunogen which comprises a bacterial toxin, means a level of circulating antibodies sufficient to protect the host from challenge with a lethal dose of the toxin.

. 5

10

15

20

25

30

As used herein the terms "protein" and "polypeptide" refer to compounds comprising amino acids joined via peptide bonds and are used interchangeably.

The terms "toxin" and "neurotoxin" when used in reference to toxins produced by members (i.e., species and strains) of the genus Clostridium are used interchangeably and refer to the proteins which are poisonous to nerve tissue.

The term "receptor-binding domain" when used in reference to a C. botulinum toxin refers to the carboxy-terminal portion of the heavy chain (H_c or the C fragment) of the toxin which is presumed to be responsible for the binding of the active toxin (i.e., the derivative toxin comprising the H and L chains joined via disulfide bonds) to receptors on the surface of synaptosomes. The receptor-binding domain for C. botulinum type A toxin is defined herein as comprising amino acid residues 861 through 1296 of SEQ ID NO:28. The receptorbinding domain for C. botulinum type B toxin is defined herein as comprising amino acid residues 848 through 1291 of SEQ ID NO:40 (strain Eklund 17B). The receptor-binding domain of C. botulinum type C1 toxin is defined herein as comprising amino acid residues 856 through 1291 of SEQ ID NO:60. The receptor-binding domain of C. botulinum type D. toxin is defined herein as comprising amino acid residues 852 through 1276 of SEQ ID NO:66. The receptor-binding domain of C. botulinum type E toxin is defined herein as comprising amino acid residues 835 through 1250 of SEQ ID NO:50 (Beluga strain). The receptor-binding domain of C. botulinum type F toxin is defined herein as comprising amino acid residues 853 through 1274 of SEQ ID NO:71. The receptor-binding domain of C. botulinum type G toxin is defined herein as comprising amino acid residues 853 through 1297 of SEQ ID NO:77. Within a given scrotype, small variations in the primary amino acid sequence of the botulinal toxins isolated from different strains has been reported [Whelan et

- 19 -

al. (1992), supra and Minton (1995) Curr. Top. Microbiol. Immunol. 195:161-194]. The present invention contemplates fusion proteins comprising the receptor-binding domain of C. botulinum toxins from serotypes A-G including the variants found among different strains within a given serotype. The receptor-binding domains listed above are used as the prototype for each strain within a serotype. Fusion proteins containing an analogous region from a strain other than the prototype strain are encompassed by the present invention.

Fusion proteins comprising the receptor binding domain (*i.e.*, C fragment) of botulinal toxins may include amino acid residues located beyond the termini of the domains defined above. For example, the pHisBotB protein contains amino acid residues 846-1291 of SEQ ID NO:40; this fusion protein thus comprises the receptor-binding domain for *C hotulinum* type B toxin as defined above (*i.e.*, Ile-848 through Glu-1291). Similarly, pHisBotE contains amino acid residues 827-1252 of SEQ ID NO:50 and pHisBotG contains amino acid residues 851-1297 of SEQ ID NO:77. Thus, both pHisBotE and pHisBotG fusion proteins contain a few amino acids located beyond the N-terminus of the defined receptor-binding domain.

The terms "native gene" or "native gene sequences" are used to indicate DNA sequences encoding a particular gene which contain the same DNA sequences as found in the gene as isolated from nature. In contrast, "synthetic gene sequences" are DNA sequences which are used to replace the naturally occurring DNA sequences when the naturally occurring sequences cause expression problems in a given host cell. For example, naturally-occurring DNA sequences encoding codons which are rarely used in a host cell may be replaced (e.g., by site-directed mutagenesis) such that the synthetic DNA sequence represents a more frequently used codon. The native DNA sequence and the synthetic DNA sequence will preferably encode the same amino acid sequence.

SUMMARY OF THE INVENTION

5

10

15

20

25

30

The present invention relates to the production of polypeptides derived from toxins particularly in recombinant host cells. In one embodiment, the present invention provides a host cell containing a recombinant expression vector, said vector encoding a protein comprising at least a portion of a *Clostridium hotulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin. The present invention is not limited by the nature of sequences encoding portions of the *C. hotulinum* toxin. These sequences may be

derived from the native gene sequences or alternatively they may comprise synthetic gene sequences. Synthetic gene sequences are employed when expression of the native gene sequences is problematic in a given host cell (e.g., when the native gene sequences contain sequences resembling yeast transcription termination signals and the desired host cell is a yeast cell).

. 5

10

15

20

25

In one embodiment, the host cell is capable of expressing the recombinant C. hotulinum toxin protein at a level greater than or equal to 2% to 40% of the total cellular protein and preferably at a level greater than or equal to 5% of the total cellular protein. In another embodiment, the host cell is capable of expressing the recombinant C. hotulinum toxin protein as a soluble protein at a level greater than or equal to 0.25% of the total cellular protein and preferably at a level greater than or equal to 0.25% to 10% of the total cellular protein.

The present invention is not limited by the nature of the host cell employed for the production of recombinant *C. hotulinum* toxin proteins. In a preferred embodiment, the host cell is an *E. coli* cell. In another preferred embodiment, the host cell is an insect cell: particularly preferred insect host cells are *Spodoptera frugiperda* (Sf9) cells. In another preferred embodiment, the host cell is a yeast cell: particularly preferred yeast cells are *Pichia pastoris* cells.

In another embodiment, the invention provides a host cell containing a recombinant expression vector, said vector encoding a fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium hotulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin. The invention is not limited by the nature of the portion of the *Clostridium hotulinum* toxin selected. In a preferred embodiment, the portion of the toxin comprises the receptor binding domain (*i.e.*, the C fragment of the toxin). The present invention is not limited by the nature of the non-toxin protein sequence employed. In a preferred embodiment, the non-toxin protein sequence comprises a polyhistidine tract. A number of alternative fusion tags or fusion partners are known to the art (*e.g.*, MBP, GST, protein A, etc.) and may be employed for the production of fusion proteins comprising a portion of a botulinal toxin.

The present invention further provides a vaccine comprising a fusion protein, said fusion protein comprising a non-toxin protein sequence and at least a portion of a Clostridium botulinum toxin, said toxin selected from the group consisting of type B toxin and type E toxin. The vaccine may be a monovalent vaccine (i.e., containing only a toxin B fusion protein or a toxin E fusion protein), a bivalent vaccine (i.e., containing both a toxin B fusion protein and a toxin E fusion protein) or a trivalent or higher valency vaccine. In a preferred embodiment, the toxin B fusion protein and/or toxin E fusion protein is combined with a fusion protein comprising a non-toxin protein sequence and at least a portion of Clostridium botulinum type A toxin. The present invention is not limited by the nature of the portion of the Clostridium botulinum toxin selected. In a preferred embodiment, the portion of the toxin comprises the receptor binding domain (i.e., the C fragment of the toxin). The present invention is not limited by the nature of the non-toxin protein sequence employed. In a preferred embodiment, the non-toxin protein sequence comprises a poly-histidine tract. A number of alternative fusion tags or fusion partners are known to the art (e.g., MBP, GST, protein A, etc.) and may be employed for the generation of fusion proteins comprising vaccines. When a fusion partner (i.e., the non-toxin protein sequence) is employed for the production of a recombinant C. botulinal toxin protein, the fusion partner may be removed from the recombinant C. botulinal toxin protein if desired (i.e., prior to administration of the protein to a subject) using a variety of methods known to the art (e.g., digestion of fusion proteins containing FactorXa or thrombin recognition sites with the appropriate enzyme). A number of the pETHis vectors employed herein provide an N-terminal his-tag followed by a FactorXa cleavage site (see Example 28a); the botulinal C fragment sequences follow the FactorXa site and thus. FactorXa can be used to remove the his-tag from the botulinal fusion protein. In a preferred embodiment, the vaccine is substantially endotoxin-free.

25

30

20

5

10.

15

The present invention is not limited by the method employed for the generation of vaccine comprising fusion proteins comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin. The fusion proteins may be produced by recombinant DNA means using either native or synthetic gene sequences expressed in a host cell. The present invention is not limited to the production of vaccines using recombinant host cells: cell free *in vitro* transcription/translation systems may be employed for the

expression of the nucleic acid constructs encoding the fusion proteins of the present invention. An example of such a cell-free system is the commercially available TnTTM Coupled Reticulocyte Lysate System (Promega Corporation, Madison, WI). Alternatively, the fusion proteins of the present invention may be generated by synthetic means (i.e., peptide synthesis).

. 5

10

15

20

25

The present invention further provides a method of generating antibody directed against a Clostridium botulinum toxin comprising: a) providing in any order: i) an antigen comprising a fusion protein comprising a non-toxin protein sequence and at least a portion of a Clostridium hotulinum toxin, said toxin selected from the group consisting of type B toxin and type E toxin, and ii) a host; and b) immunizing the host with the antigen so as to generate an antibody. In a preferred embodiment, the antigen used to immunize the host also contains a fusion protein comprising a non-toxin protein sequence and at least a portion of Clostridium botulinum type A toxin. The present invention is not limited by the nature of the portion of the Clostridium hotulinum toxin selected. In a preferred embodiment, the portion of the toxin comprises the receptor binding domain (i.e., the C fragment of the toxin). The present invention is not limited by the nature of the non-toxin protein sequence employed. In a preferred embodiment, the non-toxin protein sequence comprises a poly-histidine tract. A number of alternative fusion tags or fusion partners are known to the art (e.g., MBP, GST, protein A, etc.) and may be employed for the generation of fusion proteins comprising vaccines. When a fusion partner (i.e., the non-toxin protein sequence) is employed for the production of a recombinant C. botulinal toxin protein, the fusion partner may be removed from the recombinant C. botulinal toxin protein if desired (i.e., prior to administration of the protein to a subject) using a variety of methods known to the art (e.g., digestion of fusion proteins containing FactorXa or thrombin recognition sites with the appropriate enzyme).

The present invention is not limited by the nature of the host employed for the production of the antibodies of the invention. In a preferred embodiment, the host is a mammal, preferably a human. The antibodies of the present invention may be generated using non-mammalian hosts such as birds, preferably chickens. In a preferred embodiment the method of the present invention further comprised the step c) of collecting the antibodies

from the host. In yet another embodiment, the method of the present invention further comprises the step d) of purifying the antibodies.

The present invention further provides antibodies raised according to the above methods.

The present invention further contemplates multivalent vaccines comprising at least two recombinant *C. botulinum* toxin proteins derived from the group consisting of *C. botulinum* serotypes A. B. C. D. E. F. and G. The invention contemplates bivalent, trivalent, quadravalent, pentavalent, heptavalent and septivalent vaccines comprising recombinant *C. botulinum* toxin proteins. Preferably the recombinant *C. botulinum* toxin protein comprises the receptor binding domain (*i.e.*, *C* fragment) of the toxin.

DESCRIPTION OF THE INVENTION

5 .

10

15

20

25

The present invention contemplates vaccinating humans and other animals with polypeptides derived from *C. botulinum* neurotoxins which are substantially endotoxin-free. These botulinal peptides are also useful for the production of antitoxin. Anti-botulinal toxin antitoxin is useful for the treatment of patients effected by or at risk of symptoms due to the action of *C. botulinum* toxins. The organisms, toxins and individual steps of the present invention are described separately below.

I. Clostridium Species, Clostridial Diseases And Associated Toxins

A preferred embodiment of the method of the present invention is directed toward obtaining antibodies against *Clostridium* species, their toxins, enzymes or other metabolic byproducts, cell wall components, or synthetic or recombinant versions of any of these compounds. It is contemplated that these antibodies will be produced by immunization of humans or other animals. It is not intended that the present invention be limited to any particular toxin or any species of organism. In one embodiment, toxins from all *Clostridium* species are contemplated as immunogens. Examples of these toxins include the neuraminidase toxin of *C. butyricum*, *C. sordellii* toxins HT and LT, toxins A, B, C, D, E, F, and G of *C. botulinum* and the numerous *C. perfringens* toxins. In one preferred embodiment, toxins A.

B. and F. of C. botulinum are contemplated as immunogens. Table 2 above lists various Clostridium species, their toxins and some antigens associated with disease.

TABLE 2
Clostridial Toxins

Organism	Toxins and Disease-Associated Antigens A. B. C., C ₃ , D. E. F. G	
C. hotulinum		
C. butyricum	Neuraminidase	
C. difficile	A. B. Enterotoxin (not A nor B). Motility Altering Factor, Low Molecular Weight Toxin, Others	
C. perfringens	α. β. ε. ι. γ. δ. ν. θ. κ. λ. μ. υ	
C. sordelli C. bifermentans	HT. LT. α, β, γ α, β, γ, δ, ε, ζ, ν, θ	
C. novyi		
C septicum	α, β, γ, δ	
C. histolyticum	α, β, γ, δ, ε plus additional enzymes	
C. chanvoci	α. β. γ. ό	

It is not intended that antibodies produced against one toxin will only be used against that toxin. It is contemplated that antibodies directed against one toxin (e.g., C. perfringens type A enterotoxin) may be used as an effective therapeutic against one or more toxin(s) produced by other members of the genus Clostridium or other toxin producing organisms (e.g., Bacillus cereus, Staphylococcus aureus, Streptococcus mutans, Acinetobacter calcoaceticus, Pseudomonas aeruginosa, other Pseudomonas species, etc.). It is further contemplated that antibodies directed against the portion of the toxin which binds to mammalian membranes (e.g., C. perfringens enterotoxin A) can also be used against other organisms. It is contemplated that these membrane binding domains are produced synthetically and used as immunogens.

H. Obtaining Antibodies In Non-Mammals

5

10

1.5

20

25

30

A preferred embodiment of the method of the present invention for obtaining antibodies involves immunization. However, it is also contemplated that antibodies could be obtained from non-mammals without immunization. In the case where no immunization is

contemplated, the present invention may use non-mammals with preexisting antibodies to toxins as well as non-mammals that have antibodies to whole organisms by virtue of reactions with the administered antigen. An example of the latter involves immunization with synthetic peptides or recombinant proteins sharing epitopes with whole organism components.

5

In a preferred embodiment, the method of the present invention contemplates immunizing non-mammals with bacterial toxin(s). It is not intended that the present invention be limited to any particular toxin. In one embodiment, toxin from all clostridial bacteria sources (see Table 2) are contemplated as immunogens. Examples of these toxins are C. butyricum neuraminidase toxin, toxins A. B. C. D. E. F. and G from C. botulinum. C. perfringens toxins α, β, ε, and ι, and C. sordellii toxins HT and LT. In a preferred embodiment, C. botulinum toxins A, B, C, D, E, and F (or fragments thereof) are contemplated as immunogens.

15

10

25

20

30

A particularly preferred embodiment involves the use of bacterial toxin protein or fragments of toxin proteins produced by molecular biological means (i.e., recombinant toxin proteins). In a preferred embodiment, the immunogen comprises the receptor-binding domain (i.e., the >50 kD carboxy-terminal portion of the heavy chain; also referred to as the C fragment) of C. botulinum scrotype A neurotoxin produced by recombinant DNA technology. In another preferred embodiment, the immunogen comprises the receptor-binding domain of C. botulinum serotype B neurotoxin produced by recombinant DNA technology. In vet another preferred embodiment, the immunogen comprises the receptor-binding domain region of C. botulinum scrotype E neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of C. hotulinum scrotype C1 neurotoxin produced by recombinant DNA technology. In vet another preferred embodiment, the immunogen comprises the receptor-binding domain region of C. botulinum serotype C2 neurotoxin produced by recombinant DNA technology. In vet another preferred embodiment, the immunogen comprises the receptor-binding domain region of C. botulinum serotype D neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of C. borulinum serotype F neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of C. botulinum serotype G neurotoxin produced by recombinant DNA technology. In a preferred embodiment, the recombinant botulinal toxin proteins are expressed as fusion proteins (e.g., as histidine-tagged proteins). In a still further preferred embodiment, the

immunogen is a multivalent vaccine comprising the receptor-binding domain region of C. botulinum toxin from two or more toxins selected from the group consisting of type A. type B. type C (including C1 and C2), type D. type E, and type F toxin.

- 5

10

15

20

25

30

When immunization is used, the preferred non-mammal is from the class Aves. All birds are contemplated (e.g., duck, ostrich, emu, turkey, etc.). A preferred bird is a chicken. Importantly, chicken antibody does not fix mammalian complement. [See H.N. Benson et al., J. Immunol. 87:616 (1961).] Thus, chicken antibody will normally not cause a complement-dependent reaction. [A.A. Benedict and K. Yamaga, "Immunoglobulins and Antibody Production in Avian Species," in Comparative Immunology (J.J. Marchaloni, ed.), pp. 335-375. Blackwell, Oxford (1966).] Thus, the preferred antitoxins of the present invention will not exhibit complement-related side effects observed with antitoxins known presently.

When birds are used, it is contemplated that the antibody will be obtained from either the bird serum or the egg. A preferred embodiment involves collection of the antibody from the egg. Laying hens transport immunoglobulin to the egg yolk ("IgY") in concentrations equal to or exceeding that found in serum. [See R. Patterson et al., J. Immunol. 89:272 (1962); and S.B. Carroll and B.D. Stollar, J. Biol. Chem. 258:24 (1983).] In addition, the large volume of egg yolk produced vastly exceeds the volume of serum that can be safely obtained from the bird over any given time period. Finally, the antibody from eggs is purer and more homogeneous; there is far less non-immunoglobulin protein (as compared to serum) and only one class of immunoglobulin is transported to the yolk.

When considering immunization with toxins, one may consider modification of the toxins to reduce the toxicity. In this regard, it is not intended that the present invention be limited by immunization with modified toxin. Unmodified ("native") toxin is also contemplated as an immunogen.

It is also not intended that the present invention be limited by the type of modification -- if modification is used. The present invention contemplates all types of toxin modification, including chemical and heat treatment of the toxin. The preferred modification, however, is formaldehyde treatment.

It is not intended that the present invention be limited to a particular mode of immunization: the present invention contemplates all modes of immunization, including subcutaneous, intramuscular, intraperitoneal, and intravenous or intravascular injection, as well as *per os* administration of immunogen.

The present invention further contemplates immunization with or without adjuvant. (Adjuvant is defined as a substance known to increase the immune response to other antigens when administered with other antigens.) If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant — or that the same adjuvant, once used, be used all the time. While the present invention contemplates all types of adjuvant, whether used separately or in combinations, the preferred use of adjuvant is the use of Complete Freund's Adjuvant followed sometime later with Incomplete Freund's Adjuvant. Another preferred use of adjuvant is the use of Gerbu Adjuvant. The invention also contemplates the use of RIBI fowl adjuvant and Quil A adjuvant.

10

5

When immunization is used, the present invention contemplates a wide variety of immunization schedules. In one embodiment, a chicken is administered toxin(s) on day zero and subsequently receives toxin(s) in intervals thereafter. It is not intended that the present invention be limited by the particular intervals or doses. Similarly, it is not intended that the present invention be limited to any particular schedule for collecting antibody. The preferred collection time is sometime after day 100.

15

Where birds are used and collection of antibody is performed by collecting eggs, the eggs may be stored prior to processing for antibody. It is preferred that eggs be stored at 4°C for less than one year.

20

It is contemplated that chicken antibody produced in this manner can be bufferextracted and used analytically. While unpurified, this preparation can serve as a reference for activity of the antibody prior to further manipulations (e.g., immunoaffinity purification).

III. Increasing The Effectiveness Of Antibodies

25

When purification is used, the present invention contemplates purifying to increase the effectiveness of both non-mammalian antitoxins and mammalian antitoxins. Specifically, the present invention contemplates increasing the percent of toxin-reactive immunoglobulin. The preferred purification approach for avian antibody is polyethylene glycol (PEG) separation.

30

The present invention contemplates that avian antibody be initially purified using simple, inexpensive procedures. In one embodiment, chicken antibody from eggs is purified by extraction and precipitation with PEG. PEG purification exploits the differential solubility of lipids (which are abundant in egg yolks) and yolk proteins in high concentrations of PEG 8000. [Polson et al., Immunol. Comm. 9:495 (1980).] The technique is rapid, simple, and relatively inexpensive and yields an immunoglobulin fraction that is significantly purer in

terms of contaminating non-immunoglobulin proteins than the comparable ammonium sulfate fractions of mammalian sera and horse antibodies. The majority of the PEG is removed from the precipitated chicken immunoglobulin by treatment with ethanol. Indeed, PEG-purified antibody is sufficiently pure that the present invention contemplates the use of PEG-purified antitoxins in the passive immunization of intoxicated humans and animals.

IV. Treatment

- 5

10

15

20

25

30

The present invention contemplates antitoxin therapy for humans and other animals intoxicated by bacterial toxins. A preferred method of treatment is by intravenous administration of anti-boutlinal antitoxin; oral administration is also contemplated for other clostridial antitoxins.

A. Dosage Of Antitoxin

It was noted by way of background that a balance must be struck when administering currently available antitoxin which is usually produced in large animals such as horses: sufficient antitoxin must be administered to neutralize the toxin, but not so much antitoxin as to increase the risk of untoward side effects. These side effects are caused by: i) patient sensitivity to foreign (e.g. horse) proteins: ii) anaphylactic or immunogenic properties of non-immunoglobulin proteins: iii) the complement fixing properties of mammalian antibodies: and/or iv) the overall burden of foreign protein administered. It is extremely difficult to strike this balance when, as noted above, the degree of intoxication (and hence the level of antitoxin therapy needed) can only be approximated.

The present invention contemplates significantly reducing side effects so that this balance is more easily achieved. Treatment according to the present invention contemplates reducing side effects by using PEG-purified antitoxin from birds.

In one embodiment, the treatment of the present invention contemplates the use of PEG-purified antitoxin from birds. The use of yolk-derived, PEG-purified antibody as antitoxin allows for the administration of: 1) non(mammalian)-complement-fixing, avian antibody: 2) a less heterogeneous mixture of non-immunoglobulin proteins; and 3) less total protein to deliver the equivalent weight of active antibody present in currently available antitoxins. The non-mammalian source of the antitoxin makes it useful for treating patients who are sensitive to horse or other mammalian sera.

B. Delivery Of Antitoxin

5

10

15

20

25

30

Although it is not intended to limit the route of delivery, the present invention contemplates a method for antitoxin treatment of bacterial intoxication in which delivery of antitoxin is oral. In one embodiment, antitoxin is delivered in a solid form (e.g., tablets). In an alternative embodiment antitoxin is delivered in an aqueous solution. When an aqueous solution is used, the solution has sufficient ionic strength to solubilize antibody protein, yet is made palatable for oral administration. The delivery solution may also be buffered (e.g., carbonate buffer pH 9.5) which can neutralize stomach acids and stabilize the antibodies when the antibodies are administered orally. In one embodiment the delivery solution is an aqueous solution. In another embodiment the delivery solution is a nutritional formula. Preferably, the delivery solution is infant formula. Yet another embodiment contemplates the delivery of lyophilized antibody encapsulated or microencapsulated inside acid-resistant compounds.

Methods of applying enteric coatings to pharmaceutical compounds are well known to the art [companies specializing in the coating of pharmaceutical compounds are available; for example. The Coating Place (Verona, WI) and AAI (Wilmington, NC)]. Enteric coatings which are resistant to gastric fluid and whose release (i.e., dissolution of the coating to release the pharmaceutical compound) is pH dependent are commercially available [for example, the polymethacrylates Eudragit® L and Eudragit® S (Röhm GmbH)]. Eudragit® S is soluble in intestinal fluid from pH 7.0; this coating can be used to microencapsulate lyophilized antitoxin antibodies and the particles are suspended in a solution having a pH above or below pH 7.0 for oral administration. The microparticles will remain intact and undissolved until they reached the intestines where the intestinal pH would cause them to dissolve thereby releasing the antitoxin.

The invention contemplates a method of treatment which can be administered for treatment of acute intoxication. In one embodiment, antitoxin is administered orally in either a delivery solution or in tablet form, in therapeutic dosage, to a subject intoxicated by the bacterial toxin which served as immunogen for the antitoxin.

The invention also contemplates a method of treatment which can be administered prophylactically. In one embodiment, antitoxin is administered orally, in a delivery solution, in therapeutic dosage, to a subject, to prevent intoxication of the subject by the bacterial toxin which served as immunogen for the production of antitoxin. In another embodiment, antitoxin is administered orally in solid form such as tablets or as microencapsulated particles. Microencapsulation of lyophilized antibody using compounds such as Eudragit® (Rohm

GmbH) or polyethylene glycol, which dissolve at a wide range of pH units, allows the oral administration of solid antitoxin in a liquid form (i.e., a suspension) to recipients unable to tolerate administration of tablets (e.g., children or patients on feeding tubes). In one preferred embodiment the subject is a child. In another embodiment, antibody raised against whole bacterial organism is administered orally to a subject, in a delivery solution, in therapeutic dosage.

V. Vaccines Against Clostridial Species

5

10

15

20

25

30

The invention contemplates the generation of mono- and multivalent vaccines for the protection of an animal (particularly humans) against several clostridial species. Of particular interest are vaccines which stimulate the production of a humoral immune response to C. botulinum, C. tetani and C. difficile in humans. The antigens comprising the vaccine preparation may be native or recombinantly produced toxin proteins from the clostridial species listed above. When toxin proteins are used as immunogens they are generally modified to reduce the toxicity. This modification may be by chemical or genetic (i.e., recombinant DNA technology) means. In general genetic detoxification (i.e., the expression of nontoxic fragments in a host cell) is preferred as the expression of nontoxic fragments in a host cell precludes the presence of intact, active toxin in the final preparation. However, when chemical modification is desired, the preferred toxin modification is formaldehyde treatment.

The invention contemplates that recombinant C. botulinum toxin proteins be used as antigens in mono- and multivalent vaccine preparations. Soluble, substantially endotoxin-free recombinant C. botulinum toxin proteins derived from serotypes A. B and E may be used individually (i.e., as mono-valent vaccines) or in combination (i.e., as a multi-valent vaccine). In addition, the recombinant C. botulinum toxin proteins derived from serotypes A. B and E may be used in conjunction with either recombinant or native toxins or toxoids from other serotypes of C. botulinum, C. difficile and C. tetani as antigens for the preparation of these mono- and multivalent vaccines. It is contemplated that, due to the structural similarity of C. botulinum and C. tetani toxin proteins, a vaccine comprising C. difficile and botulinum toxin proteins (native or recombinant or a mixture thereof) be used to stimulate an immune response against C. botulinum, C. tetani and C. difficile.

- 31 -

The present invention further contemplates multi-valent vaccines comprising two or more botulinal toxin proteins selected from the group comprising recombinant (*. hotulinum toxin proteins derived from serotypes A. B. C (including C1 and C2). D. E. F and G.

The adverse consequences of exposure to botulinal toxin would be avoided by immunization of subjects at risk of exposure to the toxin with nontoxic preparations which confer immunity such as chemically or genetically detoxified toxin.

Vaccines which confer immunity against one or more of the toxin types A. B. E. F and G would be useful as a means of protecting humans from the deleterious effects of those C. hotulinum toxins known to affect man. Indeed as the possibility exists that humans could be exposed to any of the seven serotypes of C. hotulinum toxin (e.g., during biological warfare or the production of toxin in a laboratory setting), multivalent vaccines capable of conferring immunity against toxin types A-G (including both C1 and C2 toxins) would be useful for the protection of humans. Vaccines which confer immunity against one or more of the toxin types C. D and E would be useful for veterinary applications.

15

20

25

10

5

The botulinal neurotoxin is synthesized as a single polypeptide chain which is processed into a heavy (H: ~100 kD) and a light (L: ~50 kD) chain by cleavage with proteolytic enzymes; these two chains are held together via disulfide bonds in the active toxin (referred to as derivative toxin) [B.R. DasGupta and H. Sugiyama, Biochem, Biophys. Res. Commun. 48:108 (1972); reviewed in B.R. DasGupta, J. Physiol. 84:220 (1990). H. Sugiyama, Microbiol. Rev. 44:419 (1980) and C.L. Hatheway, Clin. Microbiol. Rev. 3:66 (1990)]. The heavy chain of the active toxin is cleaved by trypsin to produce two fragments termed H_c (also referred to as H₁ or C) and H_N (also referred to as H₂ or B). The H_C fragment (~46 kD) comprises the carboxy end of the H chain. The H_N fragment (~49 kD) comprises the animo end and remains attached to the L chain (H_NL). Neither H_C or H_NL is toxic. H_C competes with whole derivative toxin for binding to synaptosomes and therefore H_C is said to contain the receptor binding site. The H_C and H_N fragments of botulinal toxin are analogous to the fragments C and B of tetanus toxin which are produced by papain cleavage. The C fragment of tetanus toxin has been shown to be responsible for the binding of tetanus toxin to purified gangliosides and neuronal cells [Halpern and Loftus, J. Biol. Chem. 288:11188 (1993)].

30

Antisera raised against purified preparations of isolated botulinal H and L chains have been shown to protect mice against the lethal effects of the toxin; however, the effectiveness of the two antisera differ with the anti-H sera being more potent (H. Sugiyama, *supra*). While the different botulinal toxins show structural similarity to one another, the different

serotypes are reported to be immunologically distinct (i.e., sera raised against one toxin type does not cross-react to a significant degree with other types). Thus, the generation of multivalent vaccines may require the use of more than one type of toxin.

. 5.

10

15

20

25

30

C. botulinum toxin genes from all seven serotypes have been cloned and sequenced (Minton (1995), supra): in addition, partial amino acid sequence is available for a number of C. botulinum toxins isolated from different strains within a given serotype. The C. botulinum toxins contain about 1250-1300 amino acid residues. On the DNA level, the overall degree of homology between C. botulinum serotypes A, B, C, D and E toxins averages between 50 and 60% identity with a greater degree of homology being found between H chain-encoding regions than between those encoding L chains [Whelan et al. (1992) Appl. Environ.

Microbiol. 58:2345]. The degree of identity between C. botulinum toxins on the amino acid level reflects the level of DNA sequence homology. The most divergent area of DNA and amino acid sequence is found within the carboxy-terminal area of the various C. botulinum H chain genes. This portion of the toxin (i.e., H_C or the C fragment) plays a major role in cell binding. As toxin from different serotypes is thought to bind to distinct cell receptor molecules, it is not surprising that the toxins diverge significantly over this region.

Within a given serotype, small variations in the primary amino acid sequence of the botulinal toxins isolated from different strains has been reported [Whelan et al. (1992), supra and Minton (1995), supra]. The present invention contemplates fusion proteins comprising portions of C. botulinum toxins from serotypes A-G including the variants found among different strains within a given serotype. The present invention provides oligonucleotide primers which may be used to amplify the C fragment or receptor-binding region of the toxin gene from various strains of C. hotulinum serotype A, serotype B, serotype C (C1 and C2), serotype D, serotype E, serotype F and serotype G. A large number of different strains of C. hotulinum scrotype A. scrotype B. scrotype C, scrotype D scrotype E and scrotype F are available from the American Type Culture Collection (ATCC: Rockville, MD). For example, the ATCC provides the following: Type A strains: 174 (ATCC 3502), 457 (ATCC 17862), and NCTC 7272 (ATCC 19397); Type B strains: 34 (ATCC 439), 62A (ATCC 7948), NCA 213 B (ATCC 7949), 13114 (ATCC 8083), 3137 (ATCC 17780), 1347 (ATCC 17841), 2017 (ATCC 17843), 2217 (ATCC 17844), 2254 (ATCC 17845) and VP 1731 (ATCC 25765); Type C strains: 2220 (ATCC 17782), 2239 (ATCC 17783), 2223 (ATCC 17784; a type C-B strain: C-β strains produce C2 toxin), 662 (ATCC 17849; a type C-α strain; C-α strains produce mainly C1 toxin and a small amount of C2 toxin), 2021 (ATCC 17850; a type C-α

strain) and VPI 3803 (ATCC 25766); Type D strains: ATCC 9633, 2023 (ATCC 17851), and VPI 5995 (ATCC 27517); Type E strains: ATCC 43181, 36208 (ATCC 9564), 2231 (ATCC 17786), 2229 (ATCC 17852), 2279 (ATCC 17854) and 2285 (ATCC 17855) and Type F strains: 202F (ATCC 23387), VPI 4404 (ATCC 25764), VPI 2382 (ATCC 27321) and Langeland (ATCC 35415). Type G strain, 113/30 (NCFB 3012) may be obtained from the National Collection of Food Bacteria (NCFB, AFRC Institute of Food Research, Reading, United Kingdom).

5

10

15

20

25

30

Purification methods have been reported for native toxin types A. B. C. D. E. and F [reviewed in G. Sakaguchi, Pharmac. Ther. 19:165 (1983)]. As the different botulinal toxins are structurally related, the invention contemplates the expression of any of the botulinal toxins (e.g., types A-G) as soluble recombinant fusion proteins.

In particular, methods for purification of the type A botulinum neurotoxin have been developed [L.J. Moberg and H. Sugiyama, Appl. Environ, Microbiol. 35:878 (1978)]. Immunization of hens with detoxified purified protein results in the generation of neutralizing antibodies [B.S. Thalley et al., in Botulinum and Tetanus Neurotoxins, B.R. DasGupta, ed., Plenum Press, New York (1993), p. 467].

The currently available *C. hotulinum* pentavalent vaccine comprising chemically inactivated (*i.e.*, formaldehyde treated) type A, B, C, D and E toxins is not adequate. The efficacy is variable (in particular, only 78% of recipients produce protective levels of anti-type B antibodies following administration of the primary series) and immunization is painful (deep subcutaneous inoculation is required for administration), with adverse reactions being common (moderate to severe local reactions occur in approximately 6% of recipients upon initial injection: this number rises to approximately 11% of individuals who receive booster injections) [Informational Brochure for the Pentavalent (ABCDE) Botulinum Toxoid, Centers for Disease Control]. Preparation of this vaccine is dangerous as active toxin must be handled by laboratory workers.

In general, chemical detoxification of bacterial toxins using agents such as formaldehyde, glutaraldehyde or hydrogen peroxide is not optimal for the generation of vaccines or antitoxins. A delicate balance must be struck between too much and too little chemical modification. If the treatment is insufficient, the vaccine may retain residual toxicity. If the treatment is too excessive, the vaccine may lose potency due to destruction of native immunogenic determinants. Another major limitation of using botulinal toxoids for the generation of antitoxins or vaccines is the high production expense. For the above reasons,

the development of methods for the production of nontoxic but immunogenic C. hotulinum toxin proteins is desirable.

The C. botulinum and C. tetanus toxin proteins have similar structures [reviewed in E.J. Schantz and E.A. Johnson. Microbiol. Rev. 56:80 (1992)]. The carboxy-terminal 50 kD fragment of the tetanus toxin heavy chain (fragment C) is released by papain cleavage and has been shown to be non-toxic and immunogenic. Recombinant tetanus toxin fragment C has been developed as a candidate vaccine antigen [A.J. Makoff et al., Bio/Technology 7:1043 (1989)]. Mice immunized with recombinant tetanus toxin fragment C were protected from challenge with lethal doses of tetanus toxin. No studies have demonstrated that the recombinant tetanus fragment C protein confers immunity against other botulinal toxins such as the C. botulinum toxins.

5

10

15

20

25

30

Recombinant tetanus fragment C has been expressed in E. coli (A.J. Makoff et al., Bio/Technology, supra and Nucleic Acids Res. 17:10191 (1989); J.L. Halpern et al., Infect. Immun. 58:1004 (1990)], yeast [M.A. Romanos et al., Nucleic Acids Res. 19:1461 (1991)] and baculovirus [I.G. Charles et al., Infect. Immun. 59:1627 (1991)]. Synthetic tetanus toxin genes had to be constructed to facilitate expression in yeast (M.A. Romanos et al., supra) and E. coli [A.J. Makoff et al., Nucleic Acids Res., supra], due to the high A-T content of the tetanus toxin gene sequences. High A-T content is a common feature of clostridial genes (M.R. Popoff et al., Infect. Immun. 59:3673 (1991); H.F. LaPenotiere et al., in Botulinum and Tetanus Neurotoxins, B.R. DasGupta, ed., Plenum Press, New York (1993), p. 463] which creates expression difficulties in E. coli and yeast due primarily to altered codon usage frequency and fortuitous polyadenylation sites, respectively.

The C fragment of the C. hotulinum type A neurotoxin heavy chain has been evaluated as a vaccine candidate. The C. hotulinum type A neurotoxin gene has been cloned and sequenced [D.E. Thompson et al., Eur. J. Biochem. 189:73 (1990)]. The C fragment of the type A toxin was expressed as either a fusion protein comprising the botulinal C fragment fused with the maltose binding protein (MBP) or as a native protein [H.F. LaPenotiere et al., (1993) supra. H.F. LaPenotiere et al., Toxicon. 33:1383 (1995) and Middlebrook and Brown (1995). Curr. Top. Microbiol. Immunol. 195:89-122]. The plasmid construct encoding the native protein was reported to be unstable, while the fusion protein was expressed primarily in inclusion bodies as insoluble protein. Immunization of mice with crudely purified MBP fusion protein resulted in protection against IP challenge with 3 LD_{s0} doses of toxin [LaPenotiere et al., (1993) and (1995), supra]. However, this recombinant C. hotulinum type

A toxin C fragment/MBP fusion protein is not a suitable immunogen for the production of vaccines as it is expressed as an insoluble protein in *E. coli*. Furthermore, this recombinant *C. hotulinum* type A toxin C fragment/MBP fusion protein was not shown to be substantially free of endotoxin contamination. Experience with recombinant *C. hotulinum* type A toxin C fragment/MBP fusion proteins shows that the presence of the MBP on the fusion protein greatly complicates the removal of endotoxin from preparations of the recombinant fusion protein (see Ex. 24. infra). Expression of a synthetic gene encoding *C. hotulinum* type A toxin C fragment as a soluble protein excreted from insect cells has been reported [Middlebrook and Brown (1995). supra]: no details regarding the level of expression achieved or the presence of endotoxin or other pyrogens were provided. Like the insoluble protein expressed in *E. coli*, immunization with the recombinant protein produced in insect cells was reported to protect mice from challenge with *C. hotulinum* toxin A.

5

10

15

20

25

30

Inclusion body protein must be solubilized prior to purification and/or administration to a host. The harsh treatment of inclusion body protein needed to accomplish this solubilization may reduce the immunogenicity of the purified protein. Ideally, recombinant proteins to be used as vaccines are expressed as soluble proteins at high levels (*i.e.*, greater than or equal to about 0.75% of total cellular protein) in *E. coli* or other host cells (*e.g.*, yeast, insect cells, etc.). This facilitates the production and isolation of sufficient quantities of the immunogen in a highly purified form (*i.e.*, substantially free of endotoxin or other pyrogen contamination). The ability to express recombinant toxin proteins as soluble proteins in *E. coli* is advantageous due to the low cost of growth compared to insect or mammalian tissue culture cells.

The C. botulinum type B neurotoxin gene has been cloned and sequenced from two strains of C. botulinum type B [Whelan et al. (1992) Appl. Environ. Microbiol. 58:2345 (Danish strain) and Hutson et al. (1994) Curr. Microbiol. 28:101 (Eklund 17B strain)]. The nucleotide sequence of the toxin gene derived from the Eklund 17B strain (ATCC 25765) is available from the EMBL/GenBank sequence data banks under the accession number X71343: the nucleotide sequence of the coding region is listed in SEQ ID NO:39. The amino acid sequence of the C. botulinum type B neurotoxin derived from the strain Eklund 17B is listed in SEQ ID NO:40. The nucleotide sequence of the C. botulinum serotype B toxin gene derived from the Danish strain is listed in SEQ ID NO:41. The amino acid sequence of the C. botulinum type B neurotoxin derived from the Danish strain is listed in SEQ ID NO:42.

The C. botulinum type B neurotoxin gene is synthesized as a single polypeptide chain which is processed to form a dimer composed of a light and a heavy chain linked via disulfide bonds. The light chain is responsible for pharmacological activity (i.e., inhibition of the release of acetylcholine at the neuromuscular junction). The N-terminal portion of the heavy chain is thought to mediate channel formation while the C-terminal portion mediates toxin binding: the type B neurotoxin has been reported to exist as a mixture of predominantly single chain with some double chain (Whelan et al., supra). The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H_C domain. The present invention reports for the first time, the expression of the C fragment of C. botulinum type B toxin in heterologous hosts (e.g., E. coli).

. 5

10

15

20

25

30

The *C. hotulinum* type E neurotoxin gene has been cloned and sequenced from a number of different strains [Poulet *et al.* (1992) Biochem. Biophys. Res. Commun. 183:107; Whelan *et al.* (1992) Eur. J. Biochem. 204:657; and Fujii *et al.* (1993) J. Gen. Microbiol. 139:79]. The nucleotide sequence of the type E toxin gene is available from the EMBL sequence data bank under accession numbers X62089 (strain Beluga) and X62683 (strain NCTC 11219); the nucleotide sequence of the coding region (strain Beluga) is listed in SEQ ID NO:45. The amino acid sequence of the *C. hotulinum* type E neurotoxin derived from strain Beluga is listed in SEQ ID NO:46. The type E neurotoxin gene is synthesized as a single polypeptide chain which may be converted to a double-chain form (*i.e.*, a heavy chain and a light chain) by cleavage with trypsin: unlike the type A neurotoxin, the type E neurotoxin exists essentially only in the single-chain form. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H_c domain. The present invention reports for the first time, the expression of the C fragment of *C. hotulinum* type E toxin in heterologous hosts (*e.g., E. coli*).

The C. hotulinum type C1. D. F and G neurotoxin genes have been cloned and sequenced. The nucleotide and amino acid sequences of these genes and toxins are provided herein. The invention provides methods for the expression of the C fragment from each of these toxin genes in heterologous hosts and the purification of the resulting recombinant proteins.

The subject invention provides methods which allow the production of soluble ('. botulinum toxin proteins in economical host cells (e.g., E. coli). In addition the subject invention provides methods which allow the production of soluble botulinal toxin proteins in yeast and insect cells. Further, methods for the isolation of purified soluble ('. botulinum

toxin proteins which are suitable for immunization of humans and other animals are provided. These soluble, purified preparations of *C. botulinum* toxin proteins provide the basis for improved vaccine preparations and facilitate the production of antitoxin.

5

10

15

20

25

30

When recombinant clostridial toxin proteins produced in gram-negative bacteria (e.g., E. coli) are used as vaccines, they are purified to remove endotoxin prior to administration to a host animal. In order to vaccinate a host, an immunogenically-effective amount of purified substantially endotoxin-free recombinant clostridial toxin protein is administered in any of a number of physiologically acceptable carriers known to the art. When administered for the purpose of vaccination, the purified substantially endotoxin-free recombinant clostridial toxin protein may be used alone or in conjunction with known adjutants, including potassium alum, aluminum phosphate, aluminum hydroxide, Gerbu adjuvant (GmDP: C.C. Biotech Corp.), RIBI adjuvant (MPL; RIBI Immunochemical Research, Inc.), QS21 (Cambridge Biotech). The alum and aluminum-based adjutants are particularly preferred when vaccines are to be administered to humans; however, any adjuvant approved for use in humans may be employed. The route of immunization may be nasal, oral, intramuscular, intraperitoneal or subcutaneous.

The invention contemplates the use of soluble, substantially endotoxin-free preparations of fusion proteins comprising the C fragment of the C botulinum type A, B, C, D. E. F. and G toxin as vaccines. In one embodiment, the vaccine comprises the C fragment of either the C. botulinum type A, B, C, D, E, F, or G toxin and a poly-histidine tract (also called a histidine tag). In a particularly preferred embodiment, a fusion protein comprising the histidine tagged C fragment is expressed using the pET series of expression vectors (Novagen). The pET expression system utilizes a vector containing the T7 promoter which encodes the fusion protein and a host cell which can be induced to express the T7 DNA polymerase (i.e., a DE3 host strain). The production of C fragment fusion proteins containing a histidine tract is not limited to the use of a particular expression vector and host strain. Several commercially available expression vectors and host strains can be used to express the C fragment protein sequences as a fusion protein containing a histidine tract (For example, the pQE series (pQE-8, 12, 16, 17, 18, 30, 31, 32, 40, 41, 42, 50, 51, 52, 60 and 70) of expression vectors (Qiagen) which are used with the host strains M15[pREP4] (Qiagen) and SG13009[pREP4] (Qiagen) can be used to express fusion proteins containing six histidine residues at the amino-terminus of the fusion protein). Furthermore a number of commercially available expression vectors which provide a histidine tract also provide a protease cleavage

site between the histidine tract and the protein of interest (e.g., botulinal toxin sequences). Cleavage of the resulting fusion protein with the appropriate protease will remove the histidine tag from the protein of interest (e.g., botulinal toxin sequences) (see Example 28a. infra). Removal of the histidine tag may be desirable prior to administration of the recombinant botulinal toxin protein to a subject (e.g., a human).

VI. Detection Of Toxin

5

10

15

20

25

30

The invention contemplates detecting bacterial toxin in a sample. The term "sample" in the present specification and claims is used in its broadest sense. On the one hand it is meant to include a specimen or culture. On the other hand, it is meant to include both biological and environmental samples.

Biological samples may be animal, including human, fluid, solid (e.g., stool) or tissue: liquid and solid food products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The invention contemplates detecting bacterial toxin by a competitive immunoassay method that utilizes recombinant toxin A and toxin B proteins, antibodies raised against recombinant bacterial toxin proteins. A fixed amount of the recombinant toxin proteins are immobilized to a solid support (e.g., a microtiter plate) followed by the addition of a biological sample suspected of containing a bacterial toxin. The biological sample is first mixed with affinity-purified or PEG fractionated antibodies directed against the recombinant toxin protein. A reporter reagent is then added which is capable of detecting the presence of antibody bound to the immobilized toxin protein. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. If toxin is present in the sample, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of the reporter reagent. A control is employed where the antibody is not mixed with the sample. This gives the highest (or reference) signal.

The invention also contemplates detecting bacterial toxin by a "sandwich" immunoassay method that utilizes antibodies directed against recombinant bacterial toxin proteins. Affinity-purified antibodies directed against recombinant bacterial toxin proteins are immobilized to a solid support (e.g., microtiter plates). Biological samples suspected of containing bacterial toxins are then added followed by a washing step to remove substantially all unbound antitoxin. The biological sample is next exposed to the reporter substance, which binds to antitoxin and is then washed free of substantially all unbound reporter substance. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. Identification of the reporter substance in the biological tissue indicates the presence of the bacterial toxin.

It is also contemplated that bacterial toxin be detected by pouring liquids (e.g., soups and other fluid foods and feeds including nutritional supplements for humans and other animals) over immobilized antibody which is directed against the bacterial toxin. It is contemplated that the immobilized antibody will be present in or on such supports as cartridges, columns, beads, or any other solid support medium. In one embodiment, following the exposure of the liquid to the immobilized antibody, unbound toxin is substantially removed by washing. The exposure of the liquid is then exposed to a reporter substance which detects the presence of bound toxin. In a preferred embodiment the reporter substance is an enzyme. fluorescent dye, or radioactive compound attached to an antibody which is directed against the toxin (i.e., in a "sandwich" immunoassay). It is also contemplated that the detection system will be developed as necessary (e.g., the addition of enzyme substrate in enzyme systems; observation using fluorescent light for fluorescent dye systems; and quantitation of radioactivity for radioactive systems).

25

30

5

10

15

20

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the disclosure which follows, the following abbreviations apply: °C (degrees Centigrade): rpm (revolutions per minute): BBS-Tween (borate buffered saline containing Tween): BSA (bovine serum albumin): ELISA (enzyme-linked immunosorbent assay): CFA (complete Freund's adjuvant): IFA (incomplete Freund's adjuvant): IgG (immunoglobulin G): IgY (immunoglobulin Y): IM (intramuscular): IP (intraperitoneal): IV (intravenous or

intravascular); SC (subcutaneous); H.O (water); HCl (hydrochloric acid); LD₁₀₀ (lethal dose for 100% of experimental animals); aa (amino acid); HPLC (high performance liquid chromatography); kD (kilodaltons); gm (grams); µg (micrograms); ng (milligrams); ng (nanograms): μl (microliters): ml (milliliters): mm (millimeters): nm (nanometers): μm (micrometer): M (molar): mM (millimolar): MW (molecular weight): sec (seconds): min(s) (minute/minutes); hr(s) (hour/hours); MgCl₂ (magnesium chloride); NaCl (sodium chloride); Na.CO₃ (sodium carbonate): OD₂₈₀ (optical density at 280 nm): OD₆₀₀ (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); PBS [phosphate buffered saline (150 mM NaCl. 10 mM sodium phosphate buffer, pH 7.2)]; PEG (polyethylene glycol); PMSF (phenylmethylsulfonyl fluoride); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane): Ensure® (Ensure®, Ross Laboratories, Columbus OH); Enfamil® (Enfamil®, Mead Johnson); w/v (weight to volume); v/v (volume to volume); Amicon (Amicon, Inc., Beverly, MA); Amresco (Amresco, Inc., Solon, OH); ATCC (American Type Culture Collection, Rockville, MD); BBL (Baltimore Biologics Laboratory, (a division of Becton Dickinson). Cockeysville, MD): Becton Dickinson (Becton Dickinson Labware, Lincoln Park, NJ); BioRad (BioRad, Richmond, CA); Biotech (C-C Biotech Corp., Poway, CA): Charles River (Charles River Laboratories, Wilmington, MA); Cocalico (Cocalico Biologicals Inc., Reamstown, PA); CytRx (CytRx Corp., Norcross, GA); Falcon (e.g. Baxter Healtheare Corp., McGaw Park, IL and Becton Dickinson); FDA (Federal Food and Drug Administration): Fisher Biotech (Fisher Biotech, Springfield, NJ): GIBCO (Grand Island Biologic Company/BRL, Grand Island, NY); Gibco-BRL (Life Technologies, Inc., Gaithersburg, MD); Harlan Sprague Dawley (Harlan Sprague Dawley, Inc., Madison, WI); Mallinckrodt (a division of Baxter Healthcare Corp., McGaw Park, IL); Millipore (Millipore Corp., Marlborough, MA); New England Biolabs (New England Biolabs, Inc., Beverly, MA); Novagen (Novagen, Inc., Madison, WI); Pharmacia (Pharmacia, Inc., Piscataway, NJ); Qiagen (Qiagen, Chatsworth, CA): Sasco (Sasco, Omaha, NE): Showdex (Showa Denko America, Inc., New York, NY): Sigma (Sigma Chemical Co., St. Louis, MO): Sterogene (Sterogene, Inc., Arcadia, CA): Tech Lab (Tech Lab, Inc., Blacksburg, VA); and Vaxcell (Vaxcell, Inc., a subsidiary of CytRX Corp., Norcross, GA).

30

. 5

10

15

20

25

When a recombinant protein is described in the specification it is referred to in a short-hand manner by the amino acids in the toxin sequence present in the recombinant protein rounded to the nearest 10. For example, the recombinant protein pMB1850-2360 contains amino acids 1852 through 2362 of the *C. difficile* toxin B protein. The specification

gives detailed construction details for all recombinant proteins such that one skilled in the art will know precisely which amino acids are present in a given recombinant protein.

EXAMPLE 1

Production Of High-Titer Antibodies To Clostridium difficile Organisms In A Hen

Antibodies to certain pathogenic organisms have been shown to be effective in treating diseases caused by those organisms. It has not been shown whether antibodies can be raised, against Clostridium difficile, which would be effective in treating infection by this organism. Accordingly, C. difficile was tested as immunogen for production of hen antibodies.

To determine the best course for raising high-titer egg antibodies against whole C. difficile organisms, different immunizing strains and different immunizing concentrations were examined. The example involved (a) preparation of the bacterial immunogen.

(b) immunization, (c) purification of anti-bacterial chicken antibodies, and (d) detection of anti-bacterial antibodies in the purified IgY preparations.

a) Preparation Of Bacterial Immunogen

5

10

15

20

25

30

C. difficile strains 43594 (serogroup A) and 43596 (serogroup C) were originally obtained from the ATCC. These two strains were selected because they represent two of the most commonly-occurring serogroups isolated from patients with antibiotic-associated pseudomembranous colitis. [Delmee et al., J. Clin. Microbiol., 28(10):2210 (1990).] Additionally, both of these strains have been previously characterized with respect to their virulence in the Syrian hamster model for C. difficile infection. [Delmee et al., J. Med Microbiol., 33:85 (1990).]

The bacterial strains were separately cultured on brain heart infusion agar for 48 hours at 37°C in a Gas Pack 100 Jar (BBL. Cockeysville, MD) equipped with a Gas Pack Plus anaerobic envelope (BBL). Forty-eight hour cultures were used because they produce better growth and the organisms have been found to be more cross-reactive with respect to their surface antigen presentation. The greater the degree of cross-reactivity of our IgY preparations, the better the probability of a broad range of activity against different strains/serogroups. [Toma et al., J. Clin. Microbiol., 26(3):426 (1988).]

The resulting organisms were removed from the agar surface using a sterile dacron-tip swab, and were suspended in a solution containing 0.4% formaldehyde in PBS, pH 7.2. This

concentration of formaldehyde has been reported as producing good results for the purpose of preparing whole-organism immunogen suspensions for the generation of polyclonal anti-C. difficile antisera in rabbits. [Delmee et al., J. Clin. Microbiol., 21:323 (1985); Davies et al., Microbial Path., 9:141 (1990).] In this manner, two separate bacterial suspensions were prepared, one for each strain. The two suspensions were then incubated at 4°C for 1 hour. Following this period of formalin-treatment, the suspensions were centrifuged at $4.200 \times g$ for 20 min., and the resulting pellets were washed twice in normal saline. The washed pellets. which contained formalin-treated whole organisms, were resuspended in fresh normal saline such that the visual turbidity of each suspension corresponded to a #7 McFarland standard. [M.A.C. Edelstein, "Processing Clinical Specimens for Anaerobic Bacteria: Isolation and Identification Procedures," in S.M. Finegold et al (eds.).. Bailey and Scott's Diagnostic Microbiology, pp. 477-507, C.V. Mosby Co., (1990). The preparation of McFarland nephelometer standards and the corresponding approximate number of organisms for each tube are described in detail at pp. 172-173 of this volume.] Each of the two #7 suspensions was then split into two separate volumes. One volume of each suspension was volumetrically adjusted, by the addition of saline, to correspond to the visual turbidity of a #1 McFarland standard. [Id.] The #1 suspensions contained approximately 3×10^8 organisms/ml, and the #7 suspensions contained approximately 2 x 10° organisms/ml. [ld.] The four resulting concentration-adjusted suspensions of formalin-treated C. difficile organisms were considered to be "bacterial immunogen suspensions." These suspensions were used immediately after preparation for the initial immunization. [See section (b).]

. 5

10

15

20

25

30

The formalin-treatment procedure did not result in 100% non-viable bacteria in the immunogen suspensions. In order to increase the level of killing, the formalin concentration and length of treatment were both increased for subsequent immunogen preparations, as described below in Table 3. (Although viability was decreased with the stronger formalin treatment, 100% inviability of the bacterial immunogen suspensions was not reached.) Also, in subsequent immunogen preparations, the formalin solutions were prepared in normal saline instead of PBS. At day 49, the day of the fifth immunization, the excess volumes of the four previous bacterial immunogen suspensions were stored frozen at -70°C for use during all subsequent immunizations.

b) Immunization

For the initial immunization, 1.0 ml volumes of each of the four bacterial immunogen suspensions described above were separately emulsified in 1.2 ml volumes of CFA (GIBCO). For each of the four emulsified immunogen suspensions, two four-month old White Leghorn hens (pre-laying) were immunized. (It is not necessary to use pre-laying hens; actively-laying hens can also be utilized.) Each hen received a total volume of approximately 1.0 ml of a single emulsified immunogen suspension via four injections (two subcutaneous and two intramuscular) of approximately 250 µl per site. In this manner, a total of four different immunization combinations, using two hens per combination, were initiated for the purpose of evaluating both the effect of immunizing concentration on egg yolk antibody (IgY) production, and interstrain cross-reactivity of IgY raised against heterologous strains. The four immunization groups are summarized in Table 3.

TABLE 3
Immunization Groups

Group Designation	Immunizing Strain	Approximate Immunizing Dose
CD 43594.;#1	<i>C. difficile</i> strain 43594	1.5 × 10° organisms/hen
CD 43594, #7	14 11	1.0 × 10 organisms hen
CD 43596. #1	<i>C. difficile</i> strain 43596	1.5 × 10 ⁸ organisms/hen
CD 43596, #7	n 11	1.0 × 10' organisms/hen

20

25

5

10

15

The time point for the first series of immunizations was designated as "day zero." All subsequent immunizations were performed as described above except that the bacterial immunogen suspensions were emulsified using IFA (GIBCO) instead of CFA, and for the later time point immunization, the stored frozen suspensions were used instead of freshly-prepared suspensions. The immunization schedule used is listed in Table 4.

TABLE 4
Immunization Schedule

Day Of Immunization	Formalin-Treatment	Immunogen Preparation Used
0	1%. 1 br.	freshly-prepared
14	1%. overnight	" "
21	1%, overnight	и п
35	1º6. 48 hrs.	u n
19	1%. 72 hrs.	n n
70		stored frozen
85	м и	0 0
105		10 11

c) Purification Of Anti-Bacterial Chicken Antibodies

Groups of four eggs were collected per immunization group between days 80 and 84 post-initial immunization, and chicken immunoglobulin (IgY) was extracted according to a modification of the procedure of A. Polson et al., Immunol, Comm., 9:495 (1980). A gentle stream of distilled water from a squirt bottle was used to separate the yolks from the whites, and the yolks were broken by dropping them through a funnel into a graduated cylinder. The four individual yolks were pooled for each group. The pooled, broken yolks were blended with 4 volumes of egg extraction buffer to improve antibody yield (egg extraction buffer is 0.01 M sodium phosphate, 0.1 M NaCl, pH 7.5, containing 0.005% thimerosal), and PEG 8000 (Amresco) was added to a concentration of 3.5%. When all the PEG dissolved, the protein precipitates that formed were pelleted by centrifugation at $13,000 \times g$ for 10 minutes. The supernatants were decanted and filtered through cheesecloth to remove the lipid layer, and the PEG was added to the supernatants to a final concentration of 12% (the supernatants were assumed to contain 3.5% PEG). After a second centrifugation, the supernatants were discarded and the pellets were centrifuged a final time to extrude the remaining PEG. These crude IgY pellets were then dissolved in the original yolk volume of egg extraction buffer and stored at 4°C. As an additional control, a preimmune IgY solution was prepared as described above, using eggs collected from unimmunized hens.

5

10

15

20

25

30

- 45 -

d) Detection Of Anti-Bacterial Antibodies In The Purified IgY Preparati ns

5

10

15

20

25

30

In order to evaluate the relative levels of specific anti-C. difficile activity in the IgY preparations described above, a modified version of the whole-organism ELISA procedure of N.V. Padhye et al., J. Clin. Microbiol. 29:99-103 (1990) was used. Frozen organisms of both C. difficile strains described above were thawed and diluted to a concentration of approximately 1×10^7 organisms/ml using PBS, pH 7.2. In this way, two separate coating suspensions were prepared, one for each immunizing strain. Into the wells of 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were placed 100 µl volumes of the coating suspensions. In this manner, each plate well received a total of approximately 1×10^6 organisms of one strain or the other. The plates were then incubated at 4°C overnight. The next morning, the coating suspensions were decanted, and all wells were washed three times using PBS. In order to block non-specific binding sites, 100 µl of 0.5% BSA (Sigma) in PBS was then added to each well, and the plates were incubated for 2 hours at room temperature. The blocking solution was decanted, and 100 µl volumes of the IgY preparations described above were initially diluted 1:500 with a solution of 0.1% BSA in PBS, and then serially diluted in 1:5 steps. The following dilutions were placed in the wells: 1:500, 1:2,500, 1:62,5000, 4:312,500, and 1:1,562,500. The plates were again incubated for 2 hours at room temperature. Following this incubation, the IgY-containing solutions were decanted, and the wells were washed three times using BBS-Tween (0.1 M boric acid, 0.025 M sodium borate, 1.0 M NaCl, 0.1% Tween-20), followed by two washes using PBS-Tween (0.1% Tween-20), and finally, two washes using PBS only. To each well, 100 µl of a 1:750 dilution of rabbit anti-chicken IgG (whole-molecule)-alkaline phosphatase conjugate (Sigma) (diluted in 0.1% BSA in PBS) was added. The plates were again incubated for 2 hours at room temperature. The conjugate solutions were decanted and the plates were washed as described above. substituting 50 mM Na₃CO₃, pH 9.5 for the PBS in the final wash. The plates were developed by the addition of 100 µl of a solution containing 1 mg/ml para-nitrophenyl phosphate (Sigma) dissolved in 50 mM Na₅CO₅, 10 mM MgCL, pH 9.5 to each well, and incubating the plates at room temperature in the dark for 45 minutes. The absorbance of each well was measured at 410 nm using a Dynatech MR 700 plate reader. In this manner, each of the four IgY preparations described above was tested for reactivity against both of the immunizing C. difficile strains; strain-specific, as well as cross-reactive activity was determined.

Table 5 shows the results of the whole-organism ELISA. All four IgY preparations demonstrated significant levels of activity, to a dilution of 1:62,500 or greater against both of the immunizing organism strains. Therefore, antibodies raised against one strain were highly cross-reactive with the other strain, and vice versa. The immunizing concentration of organisms did not have a significant effect on organism-specific IgY production, as both concentrations produced approximately equivalent responses. Therefore, the lower immunizing concentration of approximately 1.5×10^8 organisms/hen is the preferred immunizing concentration of the two tested. The preimmune IgY preparation appeared to possess relatively low levels of C difficile-reactive activity to a dilution of 1:500, probably due to prior exposure of the animals to environmental clostridia.

5 .

10

15

An initial whole-organism ELISA was performed using IgY preparations made from single CD 43594, #1 and CD 43596, #1 eggs collected around day 50 (data not shown). Specific titers were found to be 5 to 10-fold lower than those reported in Table 5. These results demonstrate that it is possible to begin immunizing hens prior to the time that they begin to lay eggs, and to obtain high titer specific IgY from the first eggs that are laid. In other words, it is not necessary to wait for the hens to begin laying before the immunization schedule is started.

TABLE 5

Results Of The Anti-C. difficile Whole-Organism ELISA

leY Preparation	Dilution Of IgY Prep	43594-Coated Wells	43596-Coated Wells
CD 43594, #1	1:500	1.746	1.801
	1:2.500	1.092	1.670
	1:12,500	0.202	0.812
	1:62,500	0.136	0.179
	1:312,500	0.012	0.080
	1:1,562,500	0.002	0.020
CD 43594, #7	1:500	1.780	1.771
	1:2:500	1.025	1.078
	1:12:500	0.188	0.382
	1:62:500	0.052	0.132
	1:312:500	0.022	0.043
	1:1:562:500	0.005	0.024
CD 43596, #1	1:500	1.526	1.790
	1:2,500	0.832	1.477
	1:12,500	0.247	0.452
	1:62,500	0.050	0.242
	1:312,500	0.010	0.067
	1:1,562,500	0.000	0.036
CD 43596. #7	1:500	1.702	1.505
	1:2,500	0.706	0.866
	1:12,500	0.250	0.282
	1:62,500	0.039	0.078
	1:312,500	0.002	0.017
	1:1,562,500	0.000	0.010
Preimmune l <u>e</u> Y	1:500	0.142	0.309
	1:2.500	0.032	0.077
	1:12.500	0.006	0.024
	1:62.500	0.002	0.012
	1:312.500	0.004	0.010
	1:1.562.500	0.002	0.014

10

5

EXAMPLE 2

Treatment Of C. difficile Infection With Anti-C. difficile Antibody

In order to determine whether the immune IgY antibodies raised against whole C.

difficile organisms were capable of inhibiting the infection of hamsters by C. difficile.

hamsters infected by these bacteria were utilized. [Lyerly et al., Infect. Immun., 59:2215-2218 (1991).] This example involved: (a) determination of the lethal dose of C. difficile organisms: and (b) treatment of infected animals with immune antibody or control antibody in nutritional solution.

a) Determinati n Of The Lethal Dose Of C. difficile Organisms

- 5

10

15

20

25

30

Determination of the lethal dose of *C. difficile* organisms was carried out according to the model described by D.M. Lyerly *et al.*, Infect. Immun., 59:2215-2218 (1991). *C. difficile* strain ATCC 43596 (serogroup C, ATCC) was plated on BHI agar and grown anaerobically (BBL Gas Pak 100 system) at 37°C for 42 hours. Organisms were removed from the agar surface using a sterile dacron-tip swab and suspended in sterile 0.9% NaCl solution to a density of 10⁸ organisms/ml.

In order to determine the lethal dose of *C. difficile* in the presence of control antibody and nutritional formula, non-immune eggs were obtained from unimmunized hens and a 12% PEG preparation made as described in Example 1(c). This preparation was redissolved in one fourth the original yolk volume of vanilla flavor Ensure®.

Starting on day one, groups of female Golden Syrian hamsters (Harlan Sprague Dawley), 8-9 weeks old and weighing approximately 100 gm, were orally administered 1 ml of the preimmune/Ensure & formula at time zero, 2 hours, 6 hours, and 10 hours. At 1 hour, animals were orally administered 3.0 mg clindamycin HCl (Sigma) in 1 ml of water. This drug predisposes hamsters to *C. difficile* infection by altering the normal intestinal flora. On day two, the animals were given 1 ml of the preimmune IgY/Ensure® formula at time zero, 2 hours, 6 hours, and 10 hours. At 1 hour on day two, different groups of animals were inoculated orally with saline (control), or 10², 10⁴, 10⁶, or 10⁸ *C. difficile* organisms in 1 ml of saline. From days 3-12, animals were given 1 ml of the preimmune IgY/Ensure® formula three times daily and observed for the onset of diarrhea and death. Each animal was housed in an individual cage and was offered food and water *ad libitum*.

Administration of 10⁶ - 10⁸ organisms resulted in death in 3-4 days while the lower doses of 10⁷ - 10⁴ organisms caused death in 5 days. Cecal swabs taken from dead animals indicated the presence of *C. difficile*. Given the effectiveness of the 10² dose, this number of organisms was chosen for the following experiment to see if hyperimmune anti-*C. difficile* antibody could block infection.

b) Treatment Of Infected Animals With Immune Antibody Or Control Antibody In Nutritional Formula

The experiment in (a) was repeated using three groups of seven hamsters each. Group A received no clindamycin or C. difficile and was the survival control. Group B received clindamycin, 10² C. difficile organisms and preimmune IgY on the same schedule as the

animals in (a) above. Group C received clindamycin. 10² C. difficile organisms, and hyperimmune anti-C. difficile IgY on the same schedule as Group B. The anti-C difficile IgY was prepared as described in Example 1 except that the 12% PEG preparation was dissolved in one fourth the original yolk volume of Ensure®.

All animals were observed for the onset of diarrhea or other disease symptoms and death. Each animal was housed in an individual cage and was offered food and water ad libitum. The results are shown in Table 6.

TABLE 6
The Effect Of Oral Feeding Of Hyperimmune IgY Antibody on C. difficile Infection

Animal Group		Time To Diarrhea*	Time To Death	
A	pre-immune IgY only	no diarrhea	no deaths	
B	Clindamyein, C. difficile, preimmune lgY	30 hrs.	49 hrs.	
C.	Clindamycin, C. difficile, immune IgY	33 hrs.	56 hrs.	

Mean of seven animals.

5

10

15

20

25

30

Hamsters in the control group A did not develop diarrhea and remained healthy during the experimental period. Hamsters in groups B and C developed diarrheal disease. Anti-C difficile IgY did not protect the animals from diarrhea or death, all animals succumbed in the same time interval as the animals treated with preimmune IgY. Thus, while immunization with whole organisms apparently can improve sub-lethal symptoms with particular bacteria (see U.S. Patent No. 5.080.895 to H. Tokoro), such an approach does not prove to be productive to protect against the lethal effects of C. difficile.

EXAMPLE 3

Production of C. hotulinum Type A Antitoxin in Hens

In order to determine whether antibodies could be raised against the toxin produced by clostridial pathogens, which would be effective in treating clostridial diseases, antitoxin to C botulinum type A toxin was produced. This example involves: (a) toxin modification: (b) immunization: (c) antitoxin collection: (d) antigenicity assessment: and (e) assay of antitoxin titer.

a) Toxin Modification

C. botulinum type A toxoid was obtained from B. R. DasGupta. From this, the active type A neurotoxin (M.W. approximately 150 kD) was purified to greater than 99% purity, according to published methods. [B.R. DasGupta & V. Sathyamoorthy, Toxicon, 22:415 (1984).] The neurotoxin was detoxified with formaldehyde according to published methods. [B.R. Singh & B.R. DasGupta, Toxicon, 27:403 (1989).]

b) Immunization

. 5

10

15

20

25

30

C. hotulinum toxoid for immunization was dissolved in PBS (1 mg/ml) and was emulsified with an approximately equal volume of CFA (GIBCO) for initial immunization or IFA for booster immunization. On day zero, two white leghorn hens, obtained from local breeders, were each injected at multiple sites (intramuscular and subcutaneous) with 1 ml inactivated toxoid emulsified in 1 ml CFA. Subsequent booster immunizations were made according to the following schedule for day of injection and toxoid amount: days 14 and 21 - 0.5 mg; day 171 - 0.75 mg; days 394, 401, 409 - 0.25 mg. One hen received an additional booster of 0.150 mg on day 544.

c) Antitoxin Collection

Total yolk immunoglobulin (IgY) was extracted as described in Example 1(c) and the IgY pellet was dissolved in the original yolk volume of PBS with thimerosal.

d) Antigenicity Assessment

Eggs were collected from day 409 through day 423 to assess whether the toxoid was sufficiently immunogenic to raise antibody. Eggs from the two hens were pooled and antibody was collected as described in the standard PEG protocol. [Example 1(c).] Antigenicity of the botulinal toxin was assessed on Western blots. The 150 kD detoxified type A neurotoxin and unmodified, toxic, 300 kD botulinal type A complex (toxin used for intragastric route administration for animal gut neutralization experiments; see Example 6) were separated on a SDS-polyacrylamide reducing gel. The Western blot technique was performed according to the method of Towbin. [H. Towbin *et al.*, Proc. Natl. Acad. Sci. USA, 76:4350 (1979).] Ten μg samples of *C. botulinum* complex and toxoid were dissolved in SDS reducing sample buffer (1% SDS, 0.5% 2-mercaptoethanol, 50 mM Tris, pH 6.8, 10% glycerol, 0.025% w/v bromphenol blue, 10% β-mercaptoethanol), heated at 95°C for 10 min

and separated on a 1 mm thick 5% SDS-polyacrylamide gel. [K. Weber and M. Osborn." Proteins and Sodium Dodecyl Sulfate: Molecular Weight Determination on Polyacrylamide Gels and Related Procedures." in The Proteins. 3d Edition (H. Neurath & R.L. Hill. eds), pp. 179-223, (Academic Press, NY, 1975).] Part of the gel was cut off and the proteins were stained with Coomassie Blue. The proteins in the remainder of the gel were transferred to nitrocellulose using the Milliblot-SDE electro-blotting system (Millipore) according to manufacturer's directions. The nitrocellulose was temporarily stained with 10% Ponceau S [S.B. Carroll and A. Laughon. "Production and Purification of Polyalonal Antibodies to the Foreign Segment of β-galactosidase Fusion Proteins." in DNA Cloning: A Practical Approach. Vol.III. (D. Glover, ed.), pp. 89-111. IRL Press, Oxford. (1987)] to visualize the lanes, then destained by running a gentle stream of distilled water over the blot for several minutes. The nitrocellulose was immersed in PBS containing 3% BSA overnight at 4°C to block any remaining protein binding sites.

5

10

15

20

25

30

The blot was cut into strips and each strip was incubated with the appropriate primary antibody. The avian anti-C botulinum antibodies [described in (c)] and pre-immune chicken antibody (as control) were diluted 1:125 in PBS containing 1 mg/ml BSA for 2 hours at room temperature. The blots were washed with two changes each of large volumes of PBS. BBS-Tween and PBS, successively (10 min/wash). Goat anti-chicken lgG alkaline phosphatase conjugated secondary antibody (Fisher Biotech) was diluted 1:500 in PBS containing 1 mg/ml BSA and incubated with the blot for 2 hours at room temperature. The blots were washed with two changes each of large volumes of PBS and BBS-Tween, followed by one change of PBS and 0.1 M Tris-HCl, pH 9.5. Blots were developed in freshly prepared alkaline phosphatase substrate buffer (100 μg/ml nitroblue tetrazolium (Sigma), 50 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma), 5 mM MgCl₂ in 50 mM Na₂CO₃, pH 9.5).

The Western blots are shown in Figure 1. The anti-C. hotulinum IgY reacted to the toxoid to give a broad immunoreactive band at about 145-150 kD on the reducing gel. This toxoid is refractive to disulfide cleavage by reducing agents due to formalin crosslinking. The immune IgY reacted with the active toxin complex, a 97 kD C hotulinum type A heavy chain and a 53 kD light chain. The preimmune IgY was unreactive to the C hotulinum complex or toxoid in the Western blot.

c) Antitoxin Antibody Titer

- 5

10

15

20

25

30

The IgY antibody titer to *C. hotulinum* type A toxoid of eggs harvested between day 409 and 423 was evaluated by ELISA, prepared as follows. Ninety-six-well Falcon Pro-bind plates were coated overnight at 4°C with 100 µl/well toxoid [B.R. Singh & B.R. Das Gupta. Toxicon 27:403 (1989)] at 2.5 µg/ml in PBS, pH 7.5 containing 0.005% thimerosal. The following day the wells were blocked with PBS containing 1% BSA for 1 hour at 37°C. The IgY from immune or preimmune eggs was diluted in PBS containing 1% BSA and 0.05% Tween 20 and the plates were incubated for 1 hour at 37°C. The plates were washed three times with PBS containing 0.05% Tween 20 and three times with PBS alone. Alkaline phosphatase-conjugated goat-anti-chicken IgG (Fisher Biotech) was diluted 1:750 in PBS containing 1% BSA and 0.05% Tween 20, added to the plates, and incubated 1 hour at 37°C. The plates were washed as before, and p-nitrophenyl phosphate (Sigma) at 1 mg/ml in 0.05 M Na₂CO₃, pH 9.5, 10 mM MgCl₂ was added.

The results are shown in Figure 2. Chickens immunized with the toxoid generated high titers of antibody to the immunogen. Importantly, eggs from both immunized hens had significant anti-immunogen antibody titers as compared to preimmune control eggs. The anti-C boulinum IgY possessed significant activity, to a dilution of 1:93,750 or greater.

EXAMPLE 4

Preparation Of Avian Egg Yolk Immunoglobulin In An Orally Administrable Form

In order to administer avian IgY antibodies orally to experimental mice, an effective delivery formula for the IgY had to be determined. The concern was that if the crude IgY was dissolved in PBS, the saline in PBS would dehydrate the mice, which might prove harmful over the duration of the study. Therefore, alternative methods of oral administration of IgY were tested. The example involved: (a) isola-tion of immune IgY: (b) solubilization of IgY in water or PBS, including subsequent dialysis of the IgY-PBS solution with water to eliminate or reduce the salts (salt and phosphate) in the buffer; and (c) comparison of the quantity and activity of recovered IgY by absorbance at 280 nm and PAGE, and enzyme-linked immunoassay (ELISA).

a) Isolation Of Immune IgY

In order to investigate the most effective delivery formula for IgY, we used IgY which was raised against *Crotalus durissus terrificus* venom. Three eggs were collected from hens immunized with the *C. durissus terrificus* venom and IgY was extracted from the yolks using the modified Polson procedure described by Thalley and Carroll [Bio/Technology, 8:934-938 (1990)] as described in Example 1(c).

The egg yolks were separated from the whites, pooled, and blended with four volumes of PBS. Powdered PEG 8000 was added to a concentration of 3.5%. The mixture was centrifuged at 10.000 rpm for 10 minutes to pellet the precipitated protein, and the supernatant was filtered through cheesecloth to remove the lipid layer. Powdered PEG 8000 was added to the supernatant to bring the final PEG concentration to 12% (assuming a PEG concentration of 3.5% in the supernatant). The 12% PEG/IgY mixture was divided into two equal volumes and centrifuged to pellet the IgY.

15

10

5

b) Solubilization Of The IgY In Water Or PBS

One pellet was resuspended in 1/2 the original yolk volume of PBS, and the other pellet was resuspended in 1/2 the original yolk volume of water. The pellets were then centrifuged to remove any particles or insoluble material. The IgY in PBS solution dissolved readily but the fraction resuspended in water remained cloudy.

20

25

In order to satisfy anticipated sterility requirements for orally administered antibodies, the antibody solution needs to be filter-sterilized (as an alternative to heat sterilization which would destroy the antibodies). The preparation of IgY resuspended in water was too cloudy to pass through either a 0.2 or 0.45 µm membrane filter, so 10 ml of the PBS resuspended fraction was dialyzed overnight at room temperature against 250 ml of water. The following morning the dialysis chamber was emptied and refilled with 250 ml of fresh H₂O for a second dialysis. Thereafter, the yields of soluble antibody were determined at OD₂₈₀ and are compared in Table 7.

TABLE 7
Dependence Of IgY Yield On Solvents

	·
Absorbance Of 1:10 Dilution At 280 nm	Percent Recovery
1.149	100%
0.706	61%
0.885	77%
	1.149

Resuspending the pellets in PBS followed by dialysis against water recovered more antibody than directly resuspending the pellets in water (77% versus 61%). Equivalent volumes of the 1gY preparation in PBS or water were compared by PAGE, and these results were in accordance with the absorbance values (data not shown).

e) Activity Of IgY Prepared With Different Solvents

5

10

15

20

25

30

An ELISA was performed to compare the binding activity of the IgY extracted by each procedure described above. *C. durissus terrificus* (*C.d.t.*) venom at 2.5 μg/ml in PBS was used to coat each well of a 96-well microtiter plate. The remaining protein binding sites were blocked with PBS containing 5 mg/ml BSA. Primary antibody dilutions (in PBS containing 1 mg/ml BSA) were added in duplicate. After 2 hours of incubation at room temperature, the unbound primary antibodies were removed by washing the wells with PBS. BBS-Tween, and PBS. The species specific secondary antibody (goat anti-chicken immunoglobulin alkaline-phosphatase conjugate (Sigma) was diluted 1:750 in PBS containing 1 mg/ml BSA and added to each well of the microtiter plate. After 2 hours of incubation at room temperature, the unbound secondary antibody was removed by washing the plate as before, and freshly prepared alkaline phosphatase substrate (Sigma) at 1 mg/ml in 50 mM Na₂CO₃, 10 mM MgCl₂, pH 9.5 was added to each well. The color development was measured on a Dynatech MR 700 microplate reader using a 412 nm filter. The results are shown in Table 8.

The binding assay results parallel the recovery values in Table 7, with PBS-dissolved IgY showing slightly more activity than the PBS-dissolved/H₂O dialyzed antibody. The water-dissolved antibody had considerably less binding activity than the other preparations.

EXAMPLE 5

Survival Of Antibody Activity After Passage Through The Gastrointestinal Tract

In order to determine the feasibility of oral administration of antibody, it was of interest to determine whether orally administered IgY survived passage through the gastrointestinal tract. The example involved: (a) oral administration of specific immune antibody mixed with a nutritional formula; and (b) assay of antibody activity extracted from feces.

TABLE 8
Antigen-Binding Activity Of ley Prepared With Different Solvents

Dilution	Preimmune	PBS Dissolved	H.O Dissolved	PBS/H ₂ O
1:500	0.005	1.748	1.577	1.742
1:2.500	0.004	0.644	0.349	0.606
1:12.500	0.001	0.144	0.054	0.090
1:62,500	0.001	0.025	0.007	0.016
1:312,500	0.010	0.000	0.000	0.002

a) Oral Administration Of Antibody

The IgY preparations used in this example are the same PBS-dissolved/H₂O dialyzed antivenom materials obtained in Example 4 above, mixed with an equal volume of Enfamil®. Two mice were used in this experiment, each receiving a different diet as follows:

1) water and food as usual:

5

10

15

20

25

30

2) immune IgY preparation dialyzed against water and mixed 1:1 with Enfamil®. (The mice were given the corresponding mixture as their only source of food and water).

b) Antibody Activity After Ingestion

After both mice had ingested their respective fluids, each tube was refilled with approximately 10 ml of the appropriate fluid first thing in the morning. By mid-morning there was about 4 to 5 ml of liquid left in each tube. At this point stool samples were collected from each mouse, weighed, and dissolved in approximately 500 µl PBS per 100 mg stool sample. One hundred and sixty mg of control stools (no antibody) and 99 mg of experimental stools (specific antibody) in 1.5 ml microfuge tubes were dissolved in 800 and 500 µl PBS, respectively. The samples were heated at 37°C for 10 minutes and vortexed vigorously. The experimental stools were also broken up with a narrow spatula. Each sample

was centrifuged for 5 minutes in a microfuge and the supernatants, presumably containing the antibody extracts, were collected. The pellets were saved at 2-8°C in case future extracts were needed. Because the supernatants were tinted, they were diluted five-fold in PBS containing 1 mg/ml BSA for the initial dilution in the enzyme immunoassay (ELISA). The primary extracts were then diluted five-fold serially from this initial dilution. The volume of primary extract added to each well was 190 μ l. The ELISA was performed exactly as described in Example 4.

- 5

10

15

20

25

TABLE 9

Specific Antibody Activity After Passage Through The Gastrointestinal Tract

Dilution	Preimmune IgY	Control Fecal Extract	EXP. Fecal Extract
1:5	· ()	0.000	0.032
1:25	0.016	- 0	0.016
1:125	- 0	. 0	0.009
1:625	0	0.003	0.001
1:3125	• 0	0	0.000

There was some active antibody in the feeal extract from the mouse given the specific antibody in Enfamil® formula, but it was present at a very low level. Since the samples were assayed at an initial 1:5 dilution, the binding observed could have been higher with less dilute samples. Consequently, the mice were allowed to continue ingesting either regular food and water or the specific IgY in Enfamil® formula, as appropriate, so the assay could be repeated. Another ELISA plate was coated overnight with 5 µg/ml of C.d.t. venom in PBS.

The following morning the ELISA plate was blocked with 5 mg/ml BSA, and the fecal samples were extracted as before, except that instead of heating the extracts at 37°C, the samples were kept on ice to limit proteolysis. The samples were assayed undiluted initially, and in 5X serial dilutions thereafter. Otherwise the assay was carried out as before.

TABLE 10

Specific Antibody Survives Passage Through The Gastrointestinal Tract

Dilution	Preimmune IgY	Control Extract	Exp. Extrac
undiluted	0.003	0	0.379
1:5	. 0	0	0.071
1:25	0.000	0	0.027
1:125	0.003	· 0	0.017
1:625	0.000	- 0	0.008
1:3125	0.002	0	0.002

10

5

The experiment confirmed the previous results, with the antibody activity markedly higher. The control fecal extract showed no anti-C.d.t. activity, even undiluted, while the fecal extract from the anti-C.d.t. IgY/Enfamil@-fed mouse showed considerable anti-C.d.t. activity. This experiment (and the previous experiment) clearly demonstrate that active IgY antibody survives passage through the mouse digestive tract, a finding with favorable implications for the success of IgY antibodies administered orally as a therapeutic or prophylactic.

EXAMPLE 6

20

25

30

15

In Vivo Neutralization Of Type C. botulinum

Type A Neurotoxin By Avian Antitoxin Antibody

This example demonstrated the ability of PEG-purified antitoxin, collected as described in Example 3, to neutralize the lethal effect of C, botulinum neurotoxin type A in mice. To determine the oral lethal dose (LD_{100}) of toxin A, groups of BALB/c mice were given different doses of toxin per unit body weight (average body weight of 24 grams). For oral administration, toxin A complex, which contains the neurotoxin associated with other non-toxin proteins was used. This complex is markedly more toxic than purified neurotoxin when given by the oral route. [I. Ohishi et al., Infect. Immun., $16:106\ (1977)$.] C botulinum toxin type A complex, obtained from Eric Johnson (University Of Wisconsin, Madison) was $250\ \mu g/ml$ in $50\ mM$ sodium citrate, pH 5.5, specific toxicity 3×10^7 mouse LD_{50}/mg with parenteral administration. Approximately $40-50\ ng/gm$ body weight was usually fatal within $48\ hours$ in mice maintained on conventional food and water. When mice were given a diet and libitum of only Enfamil® the concentration needed to produce lethality was approximately

2.5 times higher (125 ng/gm body weight). Botulinal toxin concentrations of approximately 200 ng/gm body weight were fatal in mice fed Enfamil® containing preimmune IgY (resuspended in Enfamil® at the original yolk volume).

The oral LD₁₀₀ of *C. hotulinum* toxin was also determined in mice that received known amounts of a mixture of preimmune IgY-Ensure® delivered orally through feeding needles. Using a 22 gauge feeding needle, mice were given 250 µl each of a preimmune IgY-Ensure® mixture (preimmune IgY dissolved in 1/4 original volk volume) I hour before and 1/2 hour and 5 hours after administering botulinal toxin. Toxin concentrations given orally ranged from approximately 12 to 312 ng/gm body weight (0.3 to 7.5 µg per mouse). Botulinal toxin complex concentration of approximately 40 ng/gm body weight (1 µg per mouse) was lethal in all mice in less than 36 hours.

5

10

15

20

25

30

Two groups of BALB/c mice, 10 per group, were each given orally a single dose of 1 µg each of botulinal toxin complex in 100 µl of 50 mM sodium citrate pH 5.5. The mice received 250 µl treatments of a mixture of either preimmune or immune 1gY in Ensure ® (1/4 original yolk volume) 1 hour before and 1/2 hour, 4 hours, and 8 hours after botulinal toxin administration. The mice received three treatments per day for two more days. The mice were observed for 96 hours. The survival and mortality are shown in Table 11.

TABLE 11
Neutralization Of Botulinal Toxin A In Vivo

Toxin Dose ng/gm	Antibody Type	Number Of Mice Alive	Number Of Mice Dead
41.6	non-immune	0	10
41.6	anti-botulinal toxin	10	0

All mice treated with the preimmune IgY-Ensure® mixture died within 46 hours post-toxin administration. The average time of death in the mice was 32 hours post toxin administration. Treatments of preimmune IgY-Ensure® mixture did not continue beyond 24 hours due to extensive paralysis of the mouth in mice of this group. In contrast, all ten mice treated with the immune anti-botulinal toxin IgY-Ensure® mixture survived past 96 hours. Only 4 mice in this group exhibited symptoms of botulism toxicity (two mice about 2 days after and two mice 4 days after toxin administration). These mice eventually died 5 and 6 days later. Six of the mice in this immune group displayed no adverse effects to the toxin and remained alive and healthy long term. Thus, the avian anti-botulinal toxin antibody demonstrated very good protection from the lethal effects of the toxin in the experimental mice.

EXAMPLE 7

Production Of An Avian Antitoxin Against Clostridium difficile Toxin A

Toxin A is a potent cytotoxin secreted by pathogenic strains of C. difficile, that plays a direct role in damaging gastrointestinal tissues. In more severe cases of C. difficile intoxication, pseudomembranous colitis can develop which may be fatal. This would be prevented by neutralizing the effects of this toxin in the gastrointestinal tract. As a first step, antibodies were produced against a portion of the toxin. The example involved: (a) conjugation of a synthetic peptide of toxin A to bovine serum albumin: (b) immunization of hens with the peptide-BSA conjugate; and (c) detection of antitoxin peptide antibodies by ELISA.

5

10

15

20

25

30

a) Conjugation Of A Synthetic Peptide Of Toxin A To Bovine Serum Albumin

The synthetic peptide CQTIDGKKYYFN-NH, (SEQ ID NO:82) was prepared commercially (Multiple Peptide Systems. San Diego, CA) and validated to be -80% pure by high-pressure liquid chromatography. The eleven amino acids following the cysteine residue represent a consensus sequence of a repeated amino acid sequence found in Toxin A. [Wren et al., Infect. Immun., 59:3151-3155 (1991).] The cysteine was added to facilitate conjugation to carrier protein.

In order to prepare the carrier for conjugation, BSA (Sigma) was dissolved in 0.01 M NaPO₄, pH 7.0 to a final concentration of 20 mg/ml and n-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS: Pierce) was dissolved in N.N-dimethyl formamide to a concentration of 5 mg/ml. MBS solution, 0.51 ml, was added to 3.25 ml of the BSA solution and incubated for 30 minutes at room temperature with stirring every 5 minutes. The MBS-activated BSA was then purified by chromatography on a Bio-Gel P-10 column (Bio-Rad; 40 ml bed volume) equilibrated with 50 mM NaPO₄, pH 7.0 buffer. Peak fractions were pooled (6.0 ml).

Lyophilized toxin A peptide (20 mg) was added to the activated BSA mixture, stirred until the peptide dissolved and incubated 3 hours at room temperature. Within 20 minutes, the reaction mixture became cloudy and precipitates formed. After 3 hours, the reaction mixture was centrifuged at $10.000 \times g$ for 10 min and the supernatant analyzed for protein content. No significant protein could be detected at 280 nm. The conjugate precipitate was

washed three times with PBS and stored at 4°C. A second conjugation was performed with 15 mg of activated BSA and 5 mg of peptide and the conjugates pooled and suspended at a peptide concentration of 10 mg/ml in 10 mM NaPO₄, pH 7.2.

b) Immunization Of Hens With Peptide Conjugate

Two hens were each initially immunized on day zero by injection into two subcutaneous and two intramuscular sites with 1 mg of peptide conjugate that was emulsified in CFA (GIBCO). The hens were boosted on day 14 and day 21 with 1 mg of peptide conjugate emulsified in IFA (GIBCO).

Ю

. 5

c) Detection Of Antitoxin Peptide Antibodies By ELISA

IgY was purified from two eggs obtained before immunization (pre-immune) and two eggs obtained 31 and 32 days after the initial immunization using PEG fractionation as described in Example 1.

15

20

Wells of a 96-well microtiter plate (Falcon Pro-Bind Assay Plate) were coated overnight at 4°C with 100 µg/ml solution of the toxin A synthetic peptide in PBS, pH 7.2 prepared by dissolving 1 mg of the peptide in 1.0 ml of H₂O and dilution of PBS. The pre-immune and immune IgY preparations were diluted in a five-fold series in a buffer containing 1% PEG 8000 and 0.1% Tween-20 (v/v) in PBS, pH 7.2. The wells were blocked for 2 hours at room temperature with 150 µl of a solution containing 5% (v/v) Carnation® nonfat dry milk and 1% PEG 8000 in PBS, pH 7.2. After incubation for 2 hours at room temperature, the wells were washed, secondary rabbit anti-chicken IgG-alkaline phosphatase (1:750) added, the wells washed again and the color development obtained as described in Example 1. The results are shown in Table 12.

25

TABLE 12

Reactivity Of IgY With Toxin Peptide

Dilution Of PEG Prep	Absorba	nce At 410 nm
	Preimmune	Immune Anti-Peptide
1:100	0.013	0.253
1:500	0.004	0.039
1:2500	0.004	0.005

30

Clearly, the immune antibodies contain titers against this repeated epitope of toxin A.

EXAMPLE 8

Production Of Avian Antitoxins Against Clostridium difficile Native Toxins A And B

To determine whether avian antibodies are effective for the neutralization of C. difficile toxins, hens were immunized using native C. difficile toxins A and B. The resulting egg yolk antibodies were then extracted and assessed for their ability to neutralize toxins A and B in vitro. The Example involved (a) preparation of the toxin immunogens, (b) immunization, (c) purification of the antitoxins, and (d) assay of toxin neutralization activity.

10

15

20

a) Preparation Of The Toxin Immunogens

Both *C. difficile* native toxins A and B, and *C. difficile* toxoids, prepared by the treatment of the native toxins with formaldehyde, were employed as immunogens. *C. difficile* toxoids A and B were prepared by a procedure which was modified from published methods (Ehrich *et al.*. Infect. Immun. 28:1041 (1980). Separate solutions (in PBS) of native *C. difficile* toxin A and toxin B (Tech Lab) were each adjusted to a concentration of 0.20 mg/ml, and formaldehyde was added to a final concentration of 0.4%. The toxin/formaldehyde solutions were then incubated at 37°C for 40 hrs. Free formaldehyde was then removed from the resulting toxoid solutions by dialysis against PBS at 4°C. In previously published reports, this dialysis step was not performed. Therefore, free formaldehyde must have been present in their toxoid preparations. The toxoid solutions were concentrated, using a Centriprep concentrator unit (Amicon), to a final toxoid concentration of 4.0 mg/ml. The two resulting preparations were designated as toxoid A and toxoid B.

25

30

C. difficile native toxins were prepared by concentrating stock solutions of toxin A and toxin B (Tech Lab. Inc), using Centriprep concentrator units (Amicon), to a final concentration of 4.0 mg/ml.

b) Immunization

The first two immunizations were performed using the toxoid A and toxoid B immunogens described above. A total of 3 different immunization combinations were employed. For the first immunization group, 0.2 ml of toxoid A was emulsified in an equal volume of Titer Max adjuvant (CytRx). Titer Max was used in order to conserve the amount of immunogen used, and to simplify the immunization procedure. This immunization group

was designated "CTA." For the second immunization group, 0.1 ml of toxoid B was emulsified in an equal volume of Titer Max adjuvant. This group was designated "CTB." For the third immunization group, 0.2 ml of toxoid A was first mixed with 0.2 ml of toxoid B, and the resulting mixture was emulsified in 0.4 ml of Titer Max adjuvant. This group was designated "CTAB." In this way, three separate immunogen emulsions were prepared, with each emulsion containing a final concentration of 2.0 mg/ml of toxoid A (CTA) or toxoid B (CTB) or a mixture of 2.0 mg/ml toxoid A and 2.0 mg/ml toxoid B (CTAB).

- 5

10

15

20

25

30

On day 0, White Leghorn hens, obtained from a local breeder, were immunized as follows: Group CTA. Four hens were immunized, with each hen receiving 200µg of toxoid A, via two intramuscular (I.M.) injections of 50µl of CTA emulsion in the breast area. Group CTB. One hen was immunized with 200µg of toxoid B, via two I.M. injections of 50µl of CTB emulsion in the breast area. Group CTAB. Four hens were immunized, with each hen receiving a mixture containing 200µg of toxoid A and 200µg of toxoid B, via two I.M. injections of 100µl of CTAB emulsion in the breast area. The second immunization was performed 5 weeks later, on day 35, exactly as described for the first immunization above.

In order to determine whether hens previously immunized with *C. difficile* toxoids could tolerate subsequent booster immunizations using native toxins, a single hen from group CTAB was immunized for a third time, this time using a mixture of the native toxin A and native toxin B described in section (a) above (these toxins were not formaldehyde-treated, and were used in their active form). This was done in order to increase the amount (titer) and affinity of specific antitoxin antibody produced by the hen over that achieved by immunizing with toxoids only. On day 62, 0.1 ml of a toxin mixture was prepared which contained 200µg of native toxin A and 200µg of native toxin B. This toxin mixture was then emulsified in 0.1 ml of Titer Max adjuvant. A single CTAB hen was then immunized with the resulting immunogen emulsion, via two I.M. injections of 100µl each, into the breast area. This hen was marked with a wing band, and observed for adverse effects for a period of approximately 1 week, after which time the hen appeared to be in good health.

Because the CTAB hen described above tolerated the booster immunization with native toxins A and B with no adverse effects, it was decided to boost the remaining hens with native toxin as well. On day 70, booster immunizations were performed as follows: **Group CTA**. A 0.2 ml volume of the 4 mg/ml native toxin A solution was emulsified in an equal volume of Titer Max adjuvant. Each of the 4 hens was then immunized with 200µg of native toxin A, as described for the toxoid A immunizations above. **Group CTB**. A 50µl volume

of the 4 mg/ml native toxin B solution was emulsified in an equal volume of Titer Max adjuvant. The hen was then immunized with 200µg of native toxin B, as described for the toxoid B immunizations above. **Group CTAB.** A 0.15 ml volume of the 4 mg/ml native toxin A solution was first mixed with a 0.15 ml volume the 4 mg/ml native toxin B solution. The resulting toxin mixture was then emulsified in 0.3 ml of Titer Max adjuvant. The 3 remaining hens (the hen with the wing band was not immunized this time) were then immunized with 200µg of native toxin A and 200µg of native toxin B as described for the toxoid A+ toxoid B immunizations (CTAB) above. On day 85, all hens received a second booster immunization using native toxins, done exactly as described for the first boost with native toxins above.

All hens tolerated both booster immunizations with native toxins with no adverse effects. As previous literature references describe the use of formaldehyde-treated toxoids, this is apparently the first time that any immunizations have been performed using native () difficile toxins.

15

20

25

30

5

10

c) Purification Of Antitoxins

Eggs were collected from the hen in group CTB 10-12 days following the second immunization with toxoid (day 35 immunization described in section (b) above), and from the hens in groups CTA and CTAB 20-21 days following the second immunization with toxoid. To be used as a pre-immune (negative) control, eggs were also collected from unimmunized hens from the same flock. Egg yolk immunoglobulin (IgY) was extracted from the 4 groups of eggs as described in Example 1 (c), and the final IgY pellets were solubilized in the original yolk volume of PBS without thimerosal. Importantly, thimerosal was excluded because it would have been toxic to the CHO cells used in the toxin neutralization assays described in section (d) below.

d) Assay Of Toxin Neutralization Activity

The toxin neutralization activity of the IgY solutions prepared in section (c) above was determined using an assay system that was modified from published methods. [Ehrich et al., Infect. Immun. 28:1041-1043 (1992); and McGee et al. Microb. Path. 12:333-341 (1992).] As additional controls, affinity-purified goat anti-C. difficile toxin A (Tech Lab) and affinity-purified goat anti-C. difficile toxin B (Tech Lab) were also assayed for toxin neutralization activity.

The IgY solutions and goat antibodies were serially diluted using F 12 medium (GIBCO) which was supplemented with 2% FCS (GIBCO)(this solution will be referred to as "medium" for the remainder of this Example). The resulting antibody solutions were then mixed with a standardized concentration of either native C. difficile toxin A (Tech Lab), or native C. difficile toxin B (Tech Lab), at the concentrations indicated below. Following incubation at 37°C for 60 min., 100μl volumes of the toxin + antibody mixtures were added to the wells of 96-well microtiter plates (Falcon Microtest III) which contained 2.5×10^4 Chinese Hamster Ovary (CHO) cells per well (the CHO cells were plated on the previous day to allow them to adhere to the plate wells). The final concentration of toxin, or dilution of antibody indicated below refers to the final test concentration of each reagent present in the respective microtiter plate wells. Toxin reference wells were prepared which contained CHO cells and toxin A or toxin B at the same concentration used for the toxin plus antibody mixtures (these wells contained no antibody). Separate control wells were also prepared which contained CHO cells and medium only. The assay plates were then incubated for 18-24 hrs. in a 37°C, humidified, 5% CO₂ incubator. On the following day, the remaining adherent (viable) cells in the plate wells were stained using 0.2% crystal violet (Mallinckrodt) dissolved in 2% ethanol, for 10 min. Excess stain was then removed by rinsing with water, and the stained cells were solubilized by adding 100µl of 1% SDS (dissolved in water) to each well. The absorbance of each well was then measured at 570 nm, and the percent cytotoxicity of each test sample or mixture was calculated using the following formula:

. 5

10

15

20

25

30

% CHO Cell Cytotoxicity =
$$[1 - (\frac{Abs. Sample}{Abs. Control})] X 100$$

Unlike previous reports which quantitate results visually by counting cell rounding by microscopy, this Example utilized spectrophotometric methods to quantitate the *C. difficile* toxin bioassay. In order to determine the toxin A neutralizing activity of the CTA, CTAB, and pre-immune 1gY preparations, as well as the affinity-purified goat antitoxin A control, dilutions of these antibodies were reacted against a 0.1µg/ml concentration of native toxin A (this is the approx, 50% cytotoxic dose of toxin A in this assay system). The results are shown in Figure 3.

Complete neutralization of toxin A occurred with the CTA IgY (antitoxin A, above) at dilutions of 1:80 and lower, while significant neutralization occurred out to the 1:320 dilution.

The CTAB IgY (antitoxin A + toxin B, above) demonstrated complete neutralization at the 1:320-1:160 and lower dilutions, and significant neutralization occurred out to the 1:1280 dilution. The commercially available affinity-purified goat antitoxin A did not completely neutralize toxin A at any of the dilutions tested, but demonstrated significant neutralization out to a dilution of 1:1.280. The preimmune IgY did not show any toxin A neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized with toxin A alone, or simultaneously with toxin A and toxin B, is an effective toxin A antitoxin.

The toxin B neutralizing activity of the CTAB and pre-immune IgY preparations, and also the affinity-purified goat antitoxin B control was determined by reacting dilutions of these antibodies against a concentration of native toxin B of 0.1 ng/ml (approximately the 50% cytotoxic dose of toxin B in the assay system). The results are shown in Figure 4.

Complete neutralization of toxin B occurred with the CTAB IgY (antitoxin A - toxin B, above) at the 1:40 and lower dilutions, and significant neutralization occurred out to the 1:320 dilution. The affinity-purified goat antitoxin B demonstrated complete neutralization at dilutions of 1:640 and lower, and significant neutralization occurred out to a dilution of 1:2.560. The preimmune IgY did not show any toxin B neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized simultaneously with toxin A and toxin B is an effective toxin B antitoxin.

In a separate study, the toxin B neutralizing activity of CTB, CTAB, and pre-immune IgY preparations was determined by reacting dilutions of these antibodies against a native toxin B concentration of 0.1µg/ml (approximately 100% cytotoxic dose of toxin B in this assay system). The results are shown in Figure 5.

Significant neutralization of toxin B occurred with the CTB IgY (antitoxin B, above) at dilutions of 1:80 and lower, while the CTAB IgY (antitoxin A + toxin B, above) was found to have significant neutralizing activity at dilutions of 1:40 and lower. The preimmune IgY did not show any toxin B neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized with toxin B alone, or simultaneously with toxin A and toxin B, is an effective toxin B antitoxin.

30

5

10

15

20

25

EXAMPLE 9

In vivo Protection Of Golden Syrian Hamsters From

C. difficile Disease By Avian Antitoxins Against C. difficile Toxins A And B

5

10

15

20

25

30

The most extensively used animal model to study *C. difficile* disease is the hamster. [Lyerly *et al.*, Infect. Immun. 47:349-352 (1992).] Several other animal models for antibiotic-induced diarrhea exist, but none mimic the human form of the disease as closely as the hamster model. [R. Fekety, "Animal Models of Antibiotic-Induced Colitis," in O. Zak and M. Sande (eds.). Experimental Models in Antimicrobial Chemotherapy. Vol. 2, pp.61-72, (1986).] In this model, the animals are first predisposed to the disease by the oral administration of an antibiotic, such as clindamycin, which alters the population of normally-occurring gastrointestinal flora (Fekety, at 61-72). Following the oral challenge of these animals with viable *C. difficile* organisms, the hamsters develop cecitis, and hemorrhage, ulceration, and inflammation are evident in the intestinal mucosa. [Lyerly *et al.*, Infect. Immun. 47:349-352 (1985).] The animals become lethargic, develop severe diarrhea, and a high percentage of them die from the disease. [Lyerly *et al.*, Infect. Immun. 47:349-352 (1985).] This model is therefore ideally suited for the evaluation of therapeutic agents designed for the treatment or prophylaxis of *C. difficile* disease.

The ability of the avian C. difficile antitoxins, described in Example 1 above, to protect hamsters from C. difficile disease was evaluated using the Golden Syrian hamster model of C. difficile infection. The Example involved (a) preparation of the avian C. difficile antitoxins, (b) in vivo protection of hamsters from C. difficile disease by treatment with avian antitoxins, and (c) long-term survival of treated hamsters.

a) Preparation Of The Avian C. difficile Antitoxins

Eggs were collected from hens in groups CTA and CTAB described in Example 1 (b) above. To be used as a pre-immune (negative) control, eggs were also purchased from a local supermarket. Egg yolk immunoglobulin (IgY) was extracted from the 3 groups of eggs as described in Example 1 (c), and the final IgY pellets were solubilized in one fourth the original yolk volume of Ensure® nutritional formula.

b) In vivo Protection Of Hamsters Against C. difficile Disease By Treatment With Avian Antitoxins

5

10

15

20

25

30

The avian C. difficile antitoxins prepared in section (a) above were evaluated for their ability to protect hamsters from C. difficile disease using an animal model system which was modified from published procedures. [Fekcty, at 61-72; Borriello et al., J. Med. Microbiol., 24:53-64 (1987); Kim et al., Infect. Immun., 55:2984-2992 (1987); Borriello et al., J. Med. Microbiol., 25:191-196 (1988): Delmee and Avesani, J. Med. Microbiol., 33:85-90 (1990): and Lyerly et al., Infect. Immun., 59:2215-2218 (1991).] For the study, three separate experimental groups were used, with each group consisting of 7 female Golden Syrian hamsters (Charles River), approximately 10 weeks old and weighing approximately 100 gms. each. The three groups were designated "CTA," "CTAB" and "Pre-immune." These designations corresponded to the antitoxin preparations with which the animals in each group were treated. Each animal was housed in an individual cage, and was offered food and water ad libitum through the entire length of the study. On day 1, each animal was orally administered 1.0 ml of one of the three antitoxin preparations (prepared in section (a) above) at the following timepoints: 0 hrs., 4 hrs., and 8 hrs. On day 2, the day 1 treatment was repeated. On day 3, at the 0 hr, timepoint, each animal was again administered antitoxin, as described above. At 1 hr., each animal was orally administered 3.0 mg of clindamycin-HCl (Sigma) in 1 ml of water. This treatment predisposed the animals to infection with C. difficile. As a control for possible endogenous C. difficile colonization, an additional animal from the same shipment (untreated) was also administered 3.0 mg of clindamycin-HCl in the same manner. This clindamycin control animal was left untreated (and uninfected) for the remainder of the study. At the 4 hr. and 8 hr. timepoints, the animals were administered antitoxin as described above. On day 4, at the 0 hr, timepoint, each animal was again administered antitoxin as described above. At 1 hr., each animal was orally challenged with 1 ml of C. difficile inoculum, which contained approx. 100 C. difficile strain 43596 organisms in sterile saline. C. difficile strain 43596, which is a serogroup C strain, was chosen because it is representative of one of the most frequently-occurring serogroups isolated from patients with antibiotic-associated pseudomembranous colitis. [Delmee et al., J. Clin. Microbiol., 28:2210-2214 (1985). In addition, this strain has been previously demonstrated to be virulent in the hamster model of infection. [Delmee and Avesani, J. Med. Microbiol., 33:85-90 (1990).] At the 4 hr. and 8 hr. timepoints, the animals were administered antitoxin as described above. On days 5 through 13, the animals were administered antitoxin 3x per day

as described for day 1 above, and observed for the onset of diarrhea and death. On the morning of day 14, the final results of the study were tabulated. These results are shown in Table 13.

Representative animals from those that died in the Pre-Immune and CTA groups were necropsied. Viable C. difficile organisms were cultured from the ceca of these animals, and the gross pathology of the gastrointestinal tracts of these animals was consistent with that expected for C. difficile disease (inflamed, distended, hemorrhagic cecum, filled with watery diarrhea-like material). In addition, the clindamycin control animal remained healthy throughout the entire study period, therefore indicating that the hamsters used in the study had not previously been colonized with endogenous C. difficile organisms prior to the start of the study. Following the final antitoxin treatment on day 13, a single surviving animal from the CTA group, and also from the CTAB group, was sacrificed and necropsied. No pathology was noted in either animal.

TABLE 13

15

5

10

Treatment Results

Treatment Group	No. Animals Surviving	No. Animals Dead
Pre-Immune		6
CTA (Antitoxin A only)	5	2
CTAB (Antitoxin A - Antitoxin B)	7	0

20

25

30

Freatment of hamsters with orally-administered toxin A and toxin B antitoxin (group CTAB) successfully protected 7 out of 7 (100%) of the animals from C. difficile disease. Treatment of hamsters with orally-administered toxin A antitoxin (group CTA) protected 5 out of 7 (71%) of these animals from C. difficile disease. Treatment using pre-immune IgY was not protective against C. difficile disease, as only 1 out of 7 (14%) of these animals survived. These results demonstrate that the avian toxin A antitoxin and the avian toxin A toxin B antitoxin effectively protected the hamsters from C. difficile disease. These results also suggest that although the neutralization of toxin A alone confers some degree of protection against C. difficile disease, in order to achieve maximal protection, simultaneous antitoxin A and antitoxin B activity is necessary.

c) Long-Term Survival Of Treated Hamsters

It has been previously reported in the literature that hamsters treated with orally-administered bovine antitoxin IgG concentrate are protected from C. difficile disease as long

as the treatment is continued, but when the treatment is stopped, the animals develop diarrhea and subsequently die within 72 hrs. [Lyerly et al., Infect. Immun., 59(6):2215-2218 (1991).]

In order to determine whether treatment of *C. difficile* disease using avian antitoxins promotes long-term survival following the discontinuation of treatment, the 4 surviving animals in group CTAB were observed for a period of 11 days (264 hrs.) following the discontinuation of antitoxin treatment described in section (b) above. All hamsters remained healthy through the entire post-treatment period. This result demonstrates that not only does treatment with avian antitoxin protect against the onset of *C. difficile* disease (*i.e.*, it is effective as a prophylactic), it also promotes long-term survival beyond the treatment period, and thus provides a lasting cure.

EXAMPLE 10

In vivo Treatment Of Established C. difficile Infection In Golden Syrian Hamsters With Avian Antitoxins Against C. difficile Toxins A And B

15

10

5

The ability of the avian *C. difficile* antitoxins, described in Example 8 above, to treat an established *C. difficile* infection was evaluated using the Golden Syrian hamster model. The Example involved (a) preparation of the avian *C. difficile* antitoxins, (b) in vivo treatment of hamsters with established *C. difficile* infection, and (c) histologic evaluation of cecal tissue.

20

a) Preparation Of The Avian C. difficile Antitoxins

Eggs were collected from hens in group CTAB described in Example 8 (b) above, which were immunized with *C. difficile* toxoids and native toxins A and B. Eggs purchased from a local supermarket were used as a pre-immune (negative) control. Egg yolk immunoglobulin (IgY) was extracted from the 2 groups of eggs as described in Example 1 (c), and the final IgY pellets were solubilized in one-fourth the original yolk volume of Ensureire nutritional formula.

25

30

b) In vivo Treatment Of Hamsters With Established C. difficile Infection

The avian C. difficile antitoxins prepared in section (a) above were evaluated for the ability to treat established C. difficile infection in hamsters using an animal model system

which was modified from the procedure which was described for the hamster protection study in Example 8(b) above.

For the study, four separate experimental groups were used, with each group consisting of 7 female Golden Syrian hamsters (Charles River), approx. 10 weeks old, weighing approximately 100 gms, each. Each animal was housed separately, and was offered food and water *ad libitum* through the entire length of the study.

. 5

10

15

20

25

30

On day 1 of the study, the animals in all four groups were each predisposed to C. difficile infection by the oral administration of 3.0 mg of clindamycin-HCl (Sigma) in 1 ml of water.

On day 2, each animal in all four groups was orally challenged with 1 ml of C. difficile inoculum, which contained approximately 100 C. difficile strain 43596 organisms in sterile saline. C. difficile strain 43596 was chosen because it is representative of one of the most frequently-occurring serogroups isolated from patients with antibiotic-associated pseudomembranous colitis. [Delmee et al., J. Clin. Microbiol., 28:2210-2214 (1990).] In addition, as this was the same C. difficile strain used in all of the previous Examples above, it was again used in order to provide experimental continuity.

On day 3 of the study (24 hrs. post-infection), treatment was started for two of the four groups of animals. Each animal of one group was orally administered 1.0 ml of the CTAB IgY preparation (prepared in section (a) above) at the following timepoints: 0 hrs., 4 hrs., and 8 hrs. The animals in this group were designated "CTAB-24." The animals in the second group were each orally administered 1.0 ml of the pre-immune IgY preparation (also prepared in section (a) above) at the same timepoints as for the CTAB group. These animals were designated "Pre-24." Nothing was done to the remaining two groups of animals on day 3.

On day 4, 48 hrs. post-infection, the treatment described for day 3 above was repeated for the CTAB-24 and Pre-24 groups, and was initiated for the remaining two groups at the same timepoints. The final two groups of animals were designated "CTAB-48" and "Pre-48" respectively.

On days 5 through 9, the animals in all four groups were administered antitoxin or pre-immune IgY, 3x per day, as described for day 4 above. The four experimental groups are summarized in Table 14.

TABLE 14
Experimental Treatment Groups

Group Designation	Experimental Treatment	
CTAB-24	Infected, treatment w/antitoxin IgY started @ 24 hrs. post-infection.	
Pre-24	Infected, treatment w/pre-immune lgY started @ 24 hrs. post-infection.	
CTAB-48	Infected, treatment w/antitoxin IgY started @ 48 hrs. post-infection.	
Pre-48	Infected, treatment w/pre-immune IgY started @ 48 hrs. post-infection.	

. 5

10

15

20

25

30

All animals were observed for the onset of diarrhea and death through the conclusion of the study on the morning of day 10. The results of this study are displayed in Table 15.

TABLE 15
Experimental Outcome--Day 10

Treatment Group	No. Animals Surviving	No. Animals Dead
CTAB-24	6	ı
Pre-24	O	7
CTAB-48	4	3
Pre-48	2	5

Eighty-six percent of the animals which began receiving treatment with antitoxin IgY at 24 hrs. post-infection (CTAB-24 above) survived, while 57% of the animals treated with antitoxin IgY starting 48 hrs. post-infection (CTAB-48 above) survived. In contrast, none of the animals receiving pre-immune IgY starting 24 hrs. post-infection (Pre-24 above) survived, and only 29% of the animals which began receiving treatment with pre-immune IgY at 48 hrs. post-infection (Pre-48 above) survived through the conclusion of the study. These results demonstrate that avian antitoxins raised against *C. difficile* toxins A and B are capable of successfully treating established *C. difficile* infections in vivo.

c) Histologic Evaluation Of Cecal Tissue

In order to further evaluate the ability of the IgY preparations tested in this study to treat established *C. difficile* infection, histologic evaluations were performed on cecal tissue specimens obtained from representative animals from the study described in section (b) above.

Immediately following death, cecal tissue specimens were removed from animals which died in the Pre-24 and Pre-48 groups. Following the completion of the study, a representative surviving animal was sacrificed and cecal tissue specimens were removed from

the CTAB-24 and CTAB-48 groups. A single untreated animal from the same shipment as those used in the study was also sacrificed and a cecal tissue specimen was removed as a normal control. All tissue specimens were fixed overnight at 4°C in 10% buffered formalin. The fixed tissues were paraffin-embedded, sectioned, and mounted on glass microscope slides. The tissue sections were then stained using hematoxylin and eosin (H and E stain), and were examined by light microscopy.

Upon examination, the tissues obtained from the CTAB-24 and CTAB-48 animals showed no pathology, and were indistinguishable from the normal control. This observation provides further evidence for the ability of avian antitoxins raised against *C. difficile* toxins A and B to effectively treat established *C. difficile* infection, and to prevent the pathologic consequences which normally occur as a result of *C. difficile* disease.

In contrast, characteristic substantial mucosal damage and destruction was observed in the tissues of the animals from the Pre-24 and Pre-48 groups which died from *C. difficile* disease. Normal tissue architecture was obliterated in these two preparations, as most of the mucosal layer was observed to have sloughed away, and there were numerous large hemorrhagic areas containing massive numbers of erythrocytes.

EXAMPLE 11

Cloning And Expression Of C. difficile Toxin A Fragments

20

25

30

~ 5

10

15

The toxin A gene has been cloned and sequenced, and shown to encode a protein of predicted MW of 308 kd. [Dove et al., Infect. Immun., 58:480-488 (1990).] Given the expense and difficulty of isolating native toxin A protein, it would be advantageous to use simple and inexpensive procaryotic expression systems to produce and purify high levels of recombinant toxin A protein for immunization purposes. Ideally, the isolated recombinant protein would be soluble in order to preserve native antigenicity, since solubilized inclusion body proteins often do not fold into native conformations. To allow ease of purification, the recombinant protein should be expressed to levels greater than 1 mg/liter of E. coli culture.

To determine whether high levels of recombinant toxin A protein can be produced in *E. coli*, fragments of the toxin A gene were cloned into various prokaryotic expression vectors, and assessed for the ability to express recombinant toxin A protein in *E. coli*. Three prokaryotic expression systems were utilized. These systems were chosen because they drive expression of either fusion (pMALc and pGEX2T) or native (pET23a-c) protein to high levels

in *E. coli.* and allow affinity purification of the expressed protein on a ligand containing column. Fusion proteins expressed from pGEX vectors bind glutathione agarose beads, and are eluted with reduced glutathione, pMAL fusion proteins bind amylose resin, and are eluted with maltose. A poly-histidine tag is present at either the N-terminal (pET16b) or C-terminal (pET23a-c) end of pET fusion proteins. This sequence specifically binds Ni₂ chelate columns, and is eluted with imidazole salts. Extensive descriptions of these vectors are available [Williams *et al.* (1995) *DNA Cloning 2: Expression Systems*. Glover and Hames, eds. IRL Press. Oxford, pp. 15-58], and will not be discussed in detail here. The Example involved (a) cloning of the toxin A gene. (b) expression of large fragments of toxin A in various prokaryotic expression systems. (c) identification of smaller toxin A gene fragments that express efficiently in *E. coli*. (d) purification of recombinant toxin A protein by affinity chromatography, and (e) demonstration of functional activity of a recombinant fragment of the toxin A gene.

a) Cloning Of The Toxin A Gene

5

10

15

20

25

30

A restriction map of the toxin A gene is shown in Figure 6. The encoded protein contains a carboxy terminal ligand binding region, containing multiple repeats of a carbohydrate binding domain. [von Eichel-Streiber and Sauerborn, Gene 96:107-113 (1990).] The toxin A gene was cloned in three pieces, by using either the polymerase chain reaction (PCR) to amplify specific regions, (regions 1 and 2, Figure 6) or by screening a constructed genomic library for a specific toxin A gene fragment (region 3, Figure 6). The sequences of the utilized PCR primers are P1: 5' GGAAATT TAGCTGCAGCATCTGAC 3' (SEQ ID NO.:1): P2: 5' TCTAGCAAATTCGCTTGT GTTGAA 3' (SEQ ID NO.:2): P3: 5' CTCGCATATAGCCATTAGACC 3' (SEQ ID NO.:3): and P4: 5'

CTATCTAGGCCTAAAGTAT 3' (SEQ ID NO.:4). These regions were cloned into prokaryotic expression vectors that express either fusion (pMALc and pGEX2T) or native (pET23a-c) protein to high levels in *E. coli*, and allow affinity purification of the expressed protein on a ligand containing column.

Clostridium difficile VPI strain 10463 was obtained from the ATCC (ATCC #43255) and grown under anaerobic conditions in brain-heart infusion medium (BBL). High molecular-weight C. difficile DNA was isolated essentially as described by Wren and Tabaqchali (1987) J. Clin. Microbiol., 25:2402, except proteinase K and sodium dodecyl sulfate (SDS) was used to disrupt the bacteria, and cetyltrimethylammonium bromide

precipitation [as described in Ausubel et al., Current Protocols in Molecular Biology (1989)] was used to remove carbohydrates from the cleared lysate. The integrity and yield of genomic DNA was assessed by comparison with a serial dilution of uncut lambda DNA after electrophoresis on an agarose gel.

- 5

10

15

20

Fragments 1 and 2 were cloned by PCR, utilizing a proofreading thermostable DNA polymerase (native pfu polymerase; Stratagene). The high fidelity of this polymerase reduces the mutation problems associated with amplification by error prone polymerases (e.g., Taq polymerase). PCR amplification was performed using the indicated PCR primers (Figure 6) in 50 µl reactions containing 10 mM Tris-HCl(8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM each dNTP, 0.2 µM each primer, and 50 ng C. difficile genomic DNA. Reactions were overlaid with 100 µl mineral oil, heated to 94°C for 4 min, 0.5 µl native pfu polymerase (Stratagene) added, and the reaction cycled 30x at 94°C for 1 min, 50°C for 1 min, 72°C for 4 min. followed by 10 min at 72°C. Duplicate reactions were pooled, chloroform extracted, and ethanol precipitated. After washing in 70% ethanol, the pellets were resuspended in 50 μl TE buffer [10 mM Tris-HCL, 1 mM EDTA pH 8.0]. Aliquots of 10µl each were restriction digested with either EcoRI/HincII (fragment 1) or EcoRI/PstI (fragment 2), and the appropriate restriction fragments were gel purified using the Prep-A-Gene kit (BioRad), and ligated to either EcoRI/Smal-restricted pGEX2T (Pharmacia) vector (fragment 1), or the EcoRI/Pstl pMAlc (New England Biolabs) vector (fragment 2). Both clones are predicted to produce in-frame fusions with either the glutathione-S-transferase protein (pGEX vector) or the maltose binding protein (pMAL vector). Recombinant clones were isolated, and confirmed by restriction digestion, using standard recombinant molecular biology techniques. [Sambrook et al., Molecular Cloning, A Laboratory Manual (1989), and designated pGA30-660 and pMA660-1100, respectively (see Figure 6 for description of the clone designations).]

25

30

Fragment 3 was cloned from a genomic library of size selected *Pst*I digested *C. difficile* genomic DNA, using standard molecular biology techniques (Sambrook *et al.*). Given that the fragment 3 internal *Pst*I site is protected from cleavage in *C. difficile* genomic DNA [Price *et al.*, Curr. Microbiol., 16:55-60 (1987)], a 4.7 kb fragment from *Pst*I restricted *C. difficile* genomic DNA was gel purified, and ligated to *Pst*I restricted, phosphatase treated pUC9 DNA. The resulting genomic library was screened with a oligonucleotide primer specific to fragment 3, and multiple independent clones were isolated. The presence of fragment 3 in several of these clones was confirmed by restriction digestion, and a clone of the indicated orientation (Figure 6) was restricted with *BamHI/Hind*III, the released fragment

purified by gel electrophoresis, and ligated into similarly restricted pET23c expression vector DNA (Novagen). Recombinant clones were isolated, and confirmed by restriction digestion. This construct is predicted to create both a predicted in frame fusion with the pET protein leader sequence, as well as a predicted C-terminal poly-histidine affinity tag, and is designated pPA1100-2680 (see Figure 6 for the clone designation).

b) Expression Of Large Fragments Of Toxin A In E. coli

5

10

15

20

25

30

Protein expression from the three expression constructs made in (a) was induced, and analyzed by Western blot analysis with an affinity purified, goat polyclonal antiserum directed against the toxin A toxoid (Tech Lab). The procedures utilized for protein induction. SDS-PAGE, and Western blot analysis are described in detail in Williams et al (1995), supra. In brief, 5 ml 2X YT (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter, pH 7.5 + 100 µg/ml ampicillin were added to cultures of bacteria (BL21 for pMAI and pGEX plasmids, and BL21(DE3)LysS for pET plasmids) containing the appropriate recombinant clone which were induced to express recombinant protein by addition of IPTG to 1 mM. Cultures were grown at 37°C, and induced when the cell density reached 0.5 OD₆₀₀. Induced protein was allowed to accumulate for two hrs after induction. Protein samples were prepared by pelleting 1 ml aliquots of bacteria by centrifugation (1 min in a microfuge), and resuspension of the pelleted bacteria in 150 µl of 2x SDS-PAGE sample buffer [Williams et al. (1995), supra]. The samples were heated to 95°C for 5 min, the cooled and 5 or 10 µl aliquots loaded on 7.5% SDS-PAGE gels. BioRad high molecular weight protein markers were also loaded, to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining gels with Coomassie blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein. Western blots, (performed as described in Example 3) which detect toxin A reactive protein in cell lysates of induced protein from the three expression constructs are shown in Figure 7. In this figure, lanes 1-3 contain cell lysates prepared from E. coli strains containing pPA1100-2860 in B121(DE3)lysE cells: lanes 4-6 contain cell lysates prepared from E. coli strains containing pPA1100-2860 in B121(DE3)lysS cells; lanes 7-9 contain cell lysates prepared from E. coli strains containing pMA30-660; lanes 10-12 contain cell lysates prepared from E. coli strains containing pMA660-1100. The lanes were probed with an affinity purified goat antitoxin A polyclonal antibody (Tech Lab). Control lysates from uninduced cells (lanes 1, 7, and 10) contain very little immunoreactive material compared to the induced samples in the remaining

lanes. The highest molecular weight band observed for each clone is consistent with the predicted size of the full length fusion protein.

- 5

10

15

20

25

30

Each construct directs expression of high molecular weight (HMW) protein that is reactive with the toxin A antibody. The size of the largest immunoreactive bands from each sample is consistent with predictions of the estimated MW of the intact fusion proteins. This demonstrates that the three fusions are in-frame, and that none of the clones contain cloning artifacts that disrupt the integrity of the encoded fusion protein. However, the Western blot demonstrates that fusion protein from the two larger constructs (pGA30-660 and pPA1100-2680) are highly degraded. Also, expression levels of toxin A proteins from these two constructs are low, since induced protein bands are not visible by Coomassie staining (not shown). Several other expression constructs that fuse large sub-regions of the toxin A gene to either pMALc or pET23a-c expression vectors, were constructed and tested for protein induction. These constructs were made by mixing gel purified restriction fragments, derived from the expression constructs shown in Figure 6, with appropriately cleaved expression vectors, ligating, and selecting recombinant clones in which the toxin A restriction fragments had ligated together and into the expression vector as predicted for in-frame fusions. The expressed toxin A interval within these constructs are shown in Figure 8, as well as the internal restriction sites utilized to make these constructs.

As used herein, the term "interval" refers to any portion (i.e., any segment of the toxin which is less than the whole toxin molecule) of a clostridial toxin. In a preferred embodiment, "interval" refers to portions of C. difficile toxins such as toxin A or toxin B. It is also contemplated that these intervals will correspond to epitopes of immunologic importance, such as antigens or immunogens against which a neutralizing antibody response is effected. It is not intended that the present invention be limited to the particular intervals or sequences described in these Examples. It is also contemplated that sub-portions of intervals (e.g., an epitope contained within one interval or which bridges multiple intervals) be used as compositions and in the methods of the present invention.

In all cases. Western blot analysis of each of these constructs with goat antitoxin A antibody (Tech Lab) detected HMW fusion protein of the predicted size (not shown). This confirms that the reading frame of each of these clones is not prematurely terminated, and is fused in the correct frame with the fusion partner. However, the Western blot analysis revealed that in all cases, the induced protein is highly degraded, and, as assessed by the absence of identifiable induced protein bands by Coomassic Blue staining, are expressed only

at low levels. These results suggest that expression of high levels of intact toxin A recombinant protein is not possible when large regions of the toxin A gene are expressed in E. coli using these expression vectors.

c) High Level Expression Of Small Toxin A Protein Fusions In E. coli

5

10

15

20

25

30

Experience indicates that expression difficulties are often encountered when large (greater than 100 kd) fragments are expressed in *E. coli*. A number of expression constructs containing smaller fragments of the toxin A gene were constructed, to determine if small regions of the gene can be expressed to high levels without extensive protein degradation. A summary of these expression constructs are shown in Figure 9. All were constructed by inframe fusions of convenient toxin A restriction fragments to either the pMALc or pET23a-c vectors. Protein preparations from induced cultures of each of these constructs were analyzed by both Coomassie Blue staining and Western analysis as in (b) above. In all cases, higher levels of intact, full length fusion proteins were observed than with the larger recombinants from section (b).

d) Purification Of Recombinant Toxin A Protein

Large scale (500 ml) cultures of each recombinant from (c) were grown, induced, and soluble and insoluble protein fractions were isolated. The soluble protein extracts were affinity chromatographed to isolate recombinant fusion protein, as described [Williams *et al.* (1994). *supra*]. In brief, extracts containing tagged pET fusions were chromatographed on a nickel chelate column, and eluted using imidazole salts as described by the distributor (Novagen). Extracts containing soluble pMAL fusion protein were prepared and chromatographed in column buffer (10 mM NaPO₄, 0.5M NaCl, 10 mM β-mercaptoethanol, pH 7.2) over an amylose resin column (New England Biolabs), and eluted with column buffer containing 10 mM maltose as described [Williams *et al.* (1995), *supra*]. When the expressed protein was found to be predominantly insoluble, insoluble protein extracts were prepared by the method described in Example 17. *infra*. The results are summarized in Table 16. Figure 10 shows the sample purifications of recombinant toxin Δ protein. In this figure, lanes 1 and 2 contain MBP fusion protein purified by affinity purification of soluble protein.

TABLE 16
Purification Of Recombinant Toxin A Protein

Clone (a)	Protein Solubility	Yield Affinity Purified Soluble Protein (h)	% Intact Soluble Fusion Protein (c)	Yield Intact Insoluble Fusion Protein
pMA30-270	Soluble	4 mg/500 mls	10%	NA
PMA30-300	Soluble	4 mg/500 mls	5-10%	NA
pMA300-660	Insoluble		NA	10 mg/500 ml
pMA660-1100	Soluble	4.5 mg/500 mls	50%	NA
pMA1100-1610	Soluble	18 mg/500 mls	10%	NA
pMA1610-1870	Both	22 mg/500 mls	90%	20 mg/500 ml
pMA1450-1870	insoluble		NA	0.2 mg/500 ml
pPA1100-1450	Soluble	0.1 mg/500 mls	90%	NA
pPA1100-1870	Soluble	0.02 mg/500 mls	90%	NA
pMA1870-2680	Both	12 mg/500 mls	80%	NA
pPa1870-2680	Insoluble		NA	10 mg/500 ml

pP = pET23 vector, pM=pMALc vector, A=toxin A.

5

10

15

20

25

30

Estimated by Coomassie staining of SDS-PAGE gels.

Lanes 3 and 4 contain MBP fusion protein purified by solubilization of insoluble inclusion bodies. The purified fusion protein samples are pMA1870-2680 (lane 1), pMA660-1100 (lane 2), pMA300-600 (lane 3) and pMA1450-1870 (lane 4).

Poor yields of affinity purified protein were obtained when poly-histidine tagged pET vectors were used to drive expression (pPA1100-1450, pP1100-1870). However, significant protein yields were obtained from pMAL expression constructs spanning the entire toxin A gene, and yields of full-length soluble fusion protein ranged from an estimated 200-400 μg/500 ml culture (pMA30-300) to greater than 20 mg/500 ml culture (pMA1610-1870). Only one interval was expressed to high levels as strictly insoluble protein (pMA300-660). Thus, although high level expression was not observed when using large expression constructs from the toxin A gene, usable levels of recombinant protein spanning the entire toxin A gene were obtainable by isolating induced protein from a series of smaller pMAL expression constructs that span the entire toxin A gene. This is the first demonstration of the feasibility of expressing recombinant toxin A protein to high levels in *E. coli*.

Based on 1.5 OD₂₅₀ = 1 mg/ml (extinction coefficient of MBP).

e) Hemagglutination Assay Using The Toxin A Recombinant Proteins

5

10

15

20

25

30

The carboxy terminal end consisting of the repeating units contains the hemagglutination activity or binding domain of C. difficile toxin A. To determine whether the expressed toxin A recombinants retain functional activity, hemagglutination assays were performed. Two toxin A recombinant proteins, one containing the binding domain as either soluble affinity purified protein (pMA1870-2680) or SDS solubilized inclusion body protein (pPA1870-2680) and soluble protein from one region outside that domain (pMA1100-1610) were tested using a described procedure. [H.C. Krivan et. al., Infect. Immun., 53:573 (1986).] Citrated rabbit red blood cells (RRBC)(Cocalico) were washed several times with Tris-buffer (0.1M Tris and 50 mM NaCl) by centrifugation at 450 x g for 10 minutes at 4° C. A 1% RRBC suspension was made from the packed cells and resuspended in Tris-buffer. Dilutions of the recombinant proteins and native toxin A (Tech Labs) were made in the Trisbuffer and added in duplicate to a round-bottomed 96-well microtiter plate in a final volume of 100 µl. To each well, 50 µl of the 1% RRBC suspension was added, mixed by gentle tapping, and incubated at 4°C for 3-4 hours. Significant hemagglutination occurred only in the recombinant proteins containing the binding domain (pMA 1870-2680) and native toxin A. The recombinant protein outside the binding domain (pMA 1100-1610) displayed no hemagglutination activity. Using equivalent protein concentrations, the hemagglutination titer for toxin A was 1:256, while titers for the soluble and insoluble recombinant proteins of the binding domain were 1:256 and about 1:5000. Clearly, the recombinant proteins tested retained functional activity and were able to bind RRBC's.

EXAMPLE 12

Functional Activity Of IgY Reactive Against Toxin A Recombinants

The expression of recombinant toxin A protein as multiple fragments in *E.coli* has demonstrated the feasibility of generating toxin A antigen through use of recombinant methodologies (Example 11). The isolation of these recombinant proteins allows the immunoreactivity of each individual subregion of the toxin A protein to be determined (*i.e.*, in a antibody pool directed against the native toxin A protein). This identifies the regions (if any) for which little or no antibody response is elicited when the whole protein is used as a immunogen. Antibodies directed against specific fragments of the toxin A protein can be

purified by affinity chromatography against recombinant toxin A protein, and tested for neutralization ability. This identifies any toxin A subregions that are essential for producing neutralizing antibodies. Comparison with the levels of immune response directed against these intervals when native toxin is used as an immunogen predicts whether potentially higher titers of neutralizing antibodies can be produced by using recombinant protein directed against a individual region, rather than the entire protein. Finally, since it is unknown whether antibodies reactive to the recombinant toxin A proteins produced in Example 11 neutralize toxin A as effectively as antibodies raised against native toxin A (Examples 9 and 10), the protective ability of a pool of antibodies affinity purified against recombinant toxin A fragments was assessed for its ability to neutralize toxin A.

This Example involved (a) epitope mapping of the toxin A protein to determine the titre of specific antibodies directed against individual subregions of the toxin A protein when native toxin A protein is used as an immunogen. (b) affinity purification of IgY reactive against recombinant proteins spanning the toxin A gene. (c) toxin A neutralization assays with affinity purified IgY reactive to recombinant toxin A protein to identify subregions of the toxin A protein that induce the production of neutralizing antibodies, and determination of whether complete neutralization of toxin A can be elicited with a mixture of antibodies reactive to recombinant toxin A protein.

a) Epitope Mapping Of The Toxin A Gene

- 5

10

15

20

25

30

The affinity purification of recombinant toxin A protein specific to defined intervals of the toxin A protein allows epitope mapping of antibody pools directed against native toxin A. This has not previously been possible, since previous expression of toxin A recombinants has been assessed only by Western blot analysis, without knowledge of the expression levels of the protein [e.g., von Eichel-Streiber et al., J. Gen. Microbiol., 135:55-64 (1989)]. Thus, high or low reactivity of recombinant toxin A protein on Western blots may reflect protein expression level differences, not immunoreactivity differences. Given that the purified recombinant protein generated in Example 11 have been quantitated, the issue of relative immunoreactivity of individual regions of the toxin A protein was precisely addressed.

For the purposes of this Example, the toxin A protein was subdivided into 6 intervals (1-6), numbered from the amino (interval 1) to the carboxyl (interval 6) termini.

The recombinant proteins corresponding to these intervals were from expression clones (see Example 11(d) for clone designations) pMA30-300 (interval 1), pMA300-660 (interval

2). pMA660-1100 (interval 3). pPA1100-1450 (interval 4). pMA1450-1870 (interval 5) and pMA1870-2680 (interval 6). These 6 clones were selected because they span the entire protein from amino acids numbered 30 through 2680, and subdivide the protein into 6 small intervals. Also, the carbohydrate binding repeat interval is contained specifically in one interval (interval 6), allowing evaluation of the immune response specifically directed against this region. Western blots of 7.5% SDS-PAGE gels, loaded and electrophoresed with defined quantities of each recombinant protein, were probed with either goat antitoxin A polyclonal antibody (Tech Lab) or chicken antitoxin A polyclonal antibody [pCTA IgY, Example 8(c)]. The blots were prepared and developed with alkaline phosphatase as previously described [Williams et al. (1995), supra]. At least 90% of all reactivity, in either goat or chicken antibody pools, was found to be directed against the ligand binding domain (interval 6). The remaining immunoreactivity was directed against all five remaining intervals, and was similar in both antibody pools, except that the chicken antibody showed a much lower reactivity against interval 2 than the goat antibody.

5

10

15

20

25

30

This clearly demonstrates that when native toxin A is used as an immunogen in goats or chickens, the bulk of the immune response is directed against the ligand binding domain of the protein, with the remaining response distributed throughout the remaining 2/3 of the protein.

b) Affinity Purification Of IgY Reactive Against Recombinant Toxin A Protein

Affinity columns, containing recombinant toxin A protein from the 6 defined intervals in (a) above, were made and used to (i) affinity purify antibodies reactive to each individual interval from the CTA IgY preparation [Example 8(c)], and (ii) deplete interval specific antibodies from the CTA IgY preparation. Affinity columns were made by coupling 1 ml of PBS-washed Actigel resin (Sterogene) with region specific protein and 1/10 final volume of Ald-coupling solution (1M sodium cyanoborohydride). The total region specific protein added to each reaction mixture was 2.7 mg (interval 1), 3 mg (intervals 2 and 3), 0.1 mg (interval 4), 0.2 mg (interval 5) and 4 mg (interval 6). Protein for intervals 1, 3, and 6 was affinity purified pMAI fusion protein in column buffer (see Example 11). Interval 4 was affinity purified poly-histidine containing pET fusion in PBS: intervals 2 and 5 were from inclusion body preparations of insoluble pMAI, fusion protein, dialyzed extensively in PBS. Aliquots of the supernatants from the coupling reactions, before and after coupling, were

assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, in all cases greater than 50% coupling efficiencies were estimated. The resins were poured into 5 ml BioRad columns, washed extensively with PBS, and stored at 4°C.

. 5 .

10

15

20

25

30

Aliquots of the CTA IgY polyclonal antibody preparation were depleted for each individual region as described below. A 20 ml sample of the CTA IgY preparation [Example 8(c)] was dialyzed extensively against 3 changes of PBS (1 liter for each dialysis), quantitated by absorbance at OD₂₈₀, and stored at 4°C. Six 1 ml aliquots of the dialyzed IgY preparation were removed, and depleted individually for each of the six intervals. Each 1 ml aliquot was passed over the appropriate affinity column, and the eluate twice reapplied to the column. The cluate was collected, and pooled with a 1 ml PBS wash. Bound antibody was cluted from the column by washing with 5 column volumes of 4 M Guanidine-HCl (in 10 mM Tris-HCl. pH 8.0). The column was reequilibrated in PBS, and the depleted antibody stock reapplied as described above. The cluate was collected, pooled with a 1 ml PBS wash, quantitated by absorbance at OD280, and stored at 4° C. In this manner, 6 aliquots of the CTA IgY preparation were individually depleted for each of the 6 toxin A intervals, by two rounds of affinity depletion. The specificity of each depleted stock was tested by Western blot analysis. Multiple 7.5% SDS-PAGE gels were loaded with protein samples corresponding to all 6 toxin A subregions. After electrophoresis, the gels were blotted, and protein transfer confirmed by Ponceau S staining [protocols described in Williams et al. (1995), supra]. After blocking the blots 1 hr at 20°C in PBS+ 0.1% Tween 20 (PBST) containing 5% milk (as a blocking buffer), 4 ml of either a 1/500 dilution of the dialyzed CTA IgY preparation in blocking buffer, or an equivalent amount of the six depleted antibody stocks (using OD₂₈₀ to standardize antibody concentration) were added and the blots incubated a further 1 hr at room temperature. The blots were washed and developed with alkaline phosphatase (using a rabbit anti-chicken alkaline phosphate conjugate as a secondary antibody) as previously described [Williams et al. (1995), supra]. In all cases, only the target interval was depleted for antibody reactivity, and at least 90% of the reactivity to the target intervals was specifically depleted.

Region specific antibody pools were isolated by affinity chromatography as described below. Ten mls of the dialyzed CTA IgY preparation were applied sequentially to each affinity column, such that a single 10 ml aliquot was used to isolate region specific antibodies specific to each of the six subregions. The columns were sequentially washed with 10 volumes of PBS, 6 volumes of BBS-Tween, 10 volumes of TBS, and eluted with 4 ml Actisep clution media (Sterogene). The eluate was dialyzed extensively against several

changes of PBS, and the affinity purified antibody collected and stored at 4°C. The volumes of the eluate increased to greater than 10 mls during dialysis in each case, due to the high viscosity of the Actisep elution media. Aliquots of each sample were 20x concentrated using Centricon 30 microconcentrators (Amicon) and stored at 4°C. The specificity of each region specific antibody pool was tested, relative to the dialyzed CTA IgY preparation, by Western blot analysis, exactly as described above, except that 4 ml samples of blocking buffer containing 100 µl region specific antibody (unconcentrated) were used instead of the depleted CTA IgY preparations. Each affinity purified antibody preparation was specific to the defined interval, except that samples purified against intervals 1-5 also reacted with interval 6. This may be due to non-specific binding to the interval 6 protein, since this protein contains the repetitive ligand binding domain which has been shown to bind antibodies nonspecifically. [Lyerly et al., Curr. Microbiol., 19:303-306 (1989).]

5

10

15

20

25

30

The reactivity of each affinity purified antibody preparation to the corresponding proteins was approximately the same as the reactivity of the 1/500 diluted dialyzed CTA IgY preparation standard. Given that the specific antibody stocks were diluted 1/40, this would indicate that the unconcentrated affinity purified antibody stocks contain 1/10-1/20 the concentration of specific antibodies relative to the starting CTA IgY preparation.

c) Toxin A Neutralization Assay Using Antibodies Reactive Toward Recombinant Toxin A Protein

The CHO toxin neutralization assay [Example 8(d)] was used to assess the ability of the depleted or enriched samples generated in (b) above to neutralize the cytotoxicity of toxin A. The general ability of affinity purified antibodies to neutralize toxin A was assessed by mixing together aliquots of all 6 concentrated stocks of the 6 affinity purified samples generated in (b) above, and testing the ability of this mixture to neutralize a toxin A concentration of 0.1 µg/ml. The results, shown in Figure 11, demonstrate almost complete neutralization of toxin A using the affinity purified (AP) mix. Some epitopes within the recombinant proteins utilized for affinity purification were probably lost when the proteins were denatured before affinity purification [by Guanidine-HCl treatment in (b) above]. Thus, the neutralization ability of antibodies directed against recombinant protein is probably underestimated using these affinity purified antibody pools. This experiment demonstrates that antibodies reactive to recombinant toxin A can neutralize cytotoxicity, suggesting that

neutralizing antibodies may be generated by using recombinant toxin A protein as immunogen.

. 5

10

15

20

25

30

In view of the observation that the recombinant expression clones of the toxin A gene divide the protein into 6 subregions, the neutralizing ability of antibodies directed against each individual region was assessed. The neutralizing ability of antibodies directed against the ligand binding domain of toxin A was determined first.

In the toxin neutralization experiment shown in Figure 11, interval 6 specific antibodies (interval 6 contains the ligand binding domain) were depleted from the dialyzed PEG preparation, and the effect on toxin neutralization assayed. Interval 6 antibodies were depleted either by utilizing the interval 6 depleted CTA IgY preparation from (b) above ("-6 aff. depleted" in Figure 11), or by addition of interval 6 protein to the CTA IgY preparation (estimated to be a 10 fold molar excess over anti-interval 6 immunoglobulin present in this preparation) to competitively compete for interval 6 protein ("-6 prot depleted" in Figure 11). In both instances, removal of interval 6 specific antibodies reduces the neutralization efficiency relative to the starting CTA IgY preparation. This demonstrates that antibodies directed against interval 6 contribute to toxin neutralization. Since interval 6 corresponds to the ligand binding domain of the protein, these results demonstrate that antibodies directed against this region in the PEG preparation contribute to the neutralization of toxin A in this assay. However, it is significant that after removal of these antibodies, the PEG preparation retains significant ability to neutralize toxin A (Figure 11). This neutralization is probably due to the action of antibodies specific to other regions of the toxin A protein, since at least 90% of the ligand binding region reactive antibodies were removed in the depleted sample prepared in (b) above. This conclusion was supported by comparison of the toxin neutralization of the affinity purified (AP) mix compared to affinity purified interval 6 antibody alone. Although some neutralization ability was observed with AP interval 6 antibodies alone, the neutralization was significantly less than that observed with the mixture of all 6 AP antibody stocks (not shown).

Given that the mix of all six affinity purified samples almost completely neutralized the cytotoxicity of toxin A (Figure 11), the relative importance of antibodies directed against toxin A intervals 1-5 within the mixture was determined. This was assessed in two ways. First, samples containing affinity purified antibodies representing 5 of the 6 intervals were prepared, such that each individual region was depleted from one sample. Figure 12 demonstrates a sample neutralization curve, comparing the neutralization ability of affinity

purified antibody mixes without interval 4 (-4) or 5 (-5) specific antibodies, relative to the mix of all 6 affinity purified antibody stocks (positive control). While the removal of interval 5 specific antibodies had no effect on toxin neutralization (or intervals 1-3, not shown), the loss of interval 4 specific antibodies significantly reduced toxin neutralization (Figure 12).

Similar results were seen in a second experiment, in which affinity purified antibodies, directed against a single region, were added to interval 6 specific antibodies, and the effects on toxin neutralization assessed. Only interval 4 specific antibodies significantly enhanced neutralization when added to interval 6 specific antibodies (Figure 13). These results demonstrate that antibodies directed against interval 4 (corresponding to clone pPA1100-1450 in Figure 9) are important for neutralization of cytotoxicity in this assay. Epitope mapping has shown that only low levels of antibodies reactive to this region are generated when native toxin A is used as an immunogen [Example 12(a)]. It is hypothesized that immunization with recombinant protein specific to this interval will elicit higher titers of neutralizing antibodies. In summary, this analysis has identified two critical regions of the toxin A protein against which neutralizing antibodies are produced, as assayed by the CHO neutralization assay.

EXAMPLE 13

Production And Evaluation Of Avian Antitoxin Against C. difficile Recombinant Toxin A Polypeptide

20

25

30

5 .

10

15

In Example 12, we demonstrated neutralization of toxin A mediated cytotoxicity by affinity purified antibodies reactive to recombinant toxin A protein. To determine whether antibodies raised against a recombinant polypeptide fragment of *C. difficile* toxin A may be effective in treating clostridial diseases, antibodies to recombinant toxin A protein representing the binding domain were generated. Two toxin A binding domain recombinant polypeptides, expressing the binding domain in either the pMALc (pMA1870-2680) or pET 23(pPA1870-2680) vector, were used as immunogens. The pMAL protein was affinity purified as a soluble product [Example 12(d)] and the pET protein was isolated as insoluble inclusion bodies [Example 12(d)] and solubilized to an immunologically active protein using a proprietary method described in a pending patent application (U.S. Patent Application Serial No. 08/129.027). This Example involves (a) immunization, (b) antitoxin collection, (c) determination of antitoxin antibody titer, (d) anti-recombinant toxin A neutralization of toxin A hemagglutination activity *in vitro*, and (e) assay of *in vitro* toxin A neutralizing activity.

a) Immunization

The soluble and the inclusion body preparations each were used separately to immunize hens. Both purified toxin A polypeptides were diluted in PBS and emulsified with approximately equal volumes of CFA for the initial immunization or IFA for subsequent booster immunizations. On day zero, for each of the recombinant preparations, two egg laying white Leghorn hens (obtained from local breeder) were each injected at multiple sites (intramuscular and subcutaneous) with 1 ml of recombinant adjuvant mixture containing approximately 0.5 to 1.5 mgs of recombinant toxin A. Booster immunizations of 1.0 mg were given on days 14 and day 28.

Ю

• 5

b) Antitoxin Collection

Total yolk immune IgY was extracted as described in the standard PEG protocol (as in Example 1) and the final IgY pellet was dissolved in sterile PBS at the original yolk volume. This material is designated "immune recombinant IgY" or "immune IgY."

15

20

25

30

c) Antitoxin Antibody Titer

To determine if the recombinant toxin A protein was sufficiently immunogenic to raise antibodies in hens, the antibody titer of a recombinant toxin A polypeptide was determined by ELISA. Eggs from both hens were collected on day 32, the yolks pooled and the antibody was isolated using PEG as described. The immune recombinant IgY antibody titer was determined for the soluble recombinant protein containing the maltose binding protein fusion generated in p-Mal (pMA1870-2680). Ninety-six well Falcon Pro-bind plates were coated overnight at 4°C with 100 μl/well of toxin A recombinant at 2.5 μg/μl in PBS containing 0.05% thimerosal. Another plate was also coated with maltose binding protein (MBP) at the same concentration, to permit comparison of antibody reactivity to the fusion partner. The next day, the wells were blocked with PBS containing 1% bovine serum albumin (BSA) for 1 hour at 37°C. IgY isolated from immune or preimmune eggs was diluted in antibody diluent (PBS containing 1% BSA and 0.05% Tween-20), and added to the blocked wells and incubated for 1 hour at 37°C. The plates were washed three times with PBS with 0.05% Tween-20, then three times with PBS. Alkaline phosphatase conjugated rabbit anti-chicken IgG (Sigma) diluted 1:1000 in antibody diluent was added to the plate, and incubated for 1 hour at 37°C. The plates were washed as before and substrate was added. [p-nitrophenyl phosphate (Sigma)] at 1 mg/ml in 0.05M Na₂CO₃, pH 9.5 and 10 mM MgCl₂. The plates

were evaluated quantitatively on a Dynatech MR 300 Micro EPA plate reader at 410 nm about 10 minutes after the addition of substrate.

5

10

15

20

25

Based on these ELISA results, high antibody titers were raised in chickens immunized with the toxin A recombinant polypeptide. The recombinant appeared to be highly immunogenic, as it was able to generate high antibody titers relatively quickly with few immunizations. Immune IgY titer directed specifically to the toxin A portion of the recombinant was higher than the immune IgY titer to its fusion partner, the maltose binding protein, and significantly higher than the preimmune IgY. ELISA titers (reciprocal of the highest dilution of IgY generating a signal) in the preimmune IgY to the MBP or the recombinant was <1:30 while the immune IgY titers to MBP and the toxin A recombinant were 1:18750 and > 1:93750 respectively. Importantly, the anti-recombinant antibody titers generated in the hens against the recombinant polypeptide is much higher, compared to antibodies to that region raised using native toxin A. The recombinant antibody titer to region 1870-2680 in the CTA antibody preparation is at least five-fold lower compared to the recombinant generated antibodies (1:18750 versus >1:93750). Thus, it appears a better immune response can be generated against a specific recombinant using that recombinant as the immunogen compared to the native toxin A.

This observation is significant, as it shows that because recombinant portions stimulate the production of antibodies, it is not necessary to use native toxin molecules to produce antitoxin preparations. Thus, the problems associated with the toxicity of the native toxin are avoided and large-scale antitoxin production is facilitated.

Toxin A has hemagglutinating activity besides cytotoxic and enterotoxin properties.

d) Anti-Recombinant Toxin A Neutralization Of Toxin A Hemagglutination Activity In Vitro

Specifically, toxin A agglutinates rabbit erythrocytes by binding to a trisaccharide (gal 1-3B1-4GlcNAc) on the cell surface. [H. Krivan et al., Infect. Immun., 53:573-581 (1986).] We examined whether the anti-recombinant toxin A (immune IgY, antibodies raised against the insoluble product expressed in pET) can neutralize the hemagglutination activity of toxin A in vitro. The hemagglutination assay procedure used was described by H.C. Krivan et al. Polyethylene glycol-fractionated immune or preimmune IgY were pre-absorbed with citrated

rabbit erythrocytes prior to performing the hemagglutination assay because we have found that IgY alone can agglutinate red blood cells. Citrated rabbit red blood cells (RRBC's)(Cocalico)

were washed twice by centrifugation at 450 x g with isotonic buffer (0.1 M Tris-HCl. 0.05 M NaCl. pH 7.2). RRBC-reactive antibodies in the IgY were removed by preparing a 10% RRBC suspension (made by adding packed cells to immune or preimmune IgY) and incubating the mixture for 1 hour at 37°C. The RRBCs were then removed by centrifugation. Neutralization of the hemagglutination activity of toxin A by antibody was tested in roundbottomed 96-well microtiter plates. Twenty-five µl of toxin A (36 µg /ml) (Tech Lab) in isotonic buffer was mixed with an equal volume of different dilutions of immune or preimmune IgY in isotonic buffer, and incubated for 15 minutes at room temperature. Then, 50 μl of a 1% RRBC suspension in isotonic buffer was added and the mixture was incubated for 3 hours at 4°C. Positive control wells containing the final concentration of 9 µg/ml of toxin A after dilution without IgY were also included. Hemagglutination activity was assessed visually, with a diffuse matrix of RRBC's coating the bottom of the well representing a positive hemagglutination reaction and a tight button of RRBC's at the bottom of the well representing a negative reaction. The anti-recombinant immune IgY neutralized toxin A hemagglutination activity, giving a neutralization titer of 1:8. However, preimmune IgY was unable to neutralize the hemagglutination ability of toxin A.

c) Assay Of In Vitro Toxin A Neutralizing Activity

- 5

10

15

20

25

30

The ability of the anti-recombinant toxin A IgY (immune IgY antibodies raised against pMA1870-2680, the soluble recombinant binding domain protein expressed in pMAL, designated as Anti-tox. A-2 in Figure 14, and referred to as recombinant region 6) and pre-immune IgY, prepared as described in Example 8(c) above, to neutralize the cytotoxic activity of toxin A was assessed *in vitro* using the CHO cell cytotoxicity assay, and toxin A (Tech Lab) at a concentration of 0.1µg/ml, as described in Example 8(d) above. As additional controls, the anti-native toxin A IgY (CTA) and pre-immune IgY preparations described in Example 8(c) above were also tested. The results are shown in Figure 14.

The anti-recombinant toxin A IgY demonstrated only partial neutralization of the cytotoxic activity of toxin A, while the pre-immune IgY did not demonstrate any significant neutralizing activity.

EXAMPLE 14

In vivo Neutralization Of C. difficile Toxin A

The ability of avian antibodies (IgY) raised against recombinant toxin A binding domain to neutralize the enterotoxin activity of C. difficile toxin A was evaluated in vivo using Golden Syrian hamsters. The Example involved: (a) preparation of the avian anti-recombinant toxin A IgY for oral administration: (b) in vivo protection of hamsters from C. difficile toxin A enterotoxicity by treatment of toxin A with avian anti-recombinant toxin A IgY: and (c) histologic evaluation of hamster eeca.

10

5

a) Preparation Of The Avian Anti-Recombinant Toxin A IgY For Oral Administration

Eggs were collected from hens which had been immunized with the recombinant C difficile toxin A fragment pMA1870-2680 (described in Example 13, above). A second group of eggs purchased at a local supermarket was used as a pre-immune (negative) control. Egg yolk immunoglobulin (IgY) was extracted by PEG from the two groups of eggs as described in Example 8(c), and the final IgY pellets were solubilized in one-fourth the original yolk volume using 0.1M carbonate buffer (mixture of NaHCO₃ and Na₃CO₄), pH 9.5. The basic carbonate buffer was used in order to protect the toxin A from the acidic pH of the stomach environment.

20

15

b) In vivo Protection Of Hamsters Against C. difficile Toxin A Enterotoxicity By Treatment Of Toxin A With Avian Antirecombinant Toxin A IgY

25

In order to assess the ability of the avian anti-recombinant toxin A IgY, prepared in section (a) above to neutralize the *in vivo* enterotoxin activity of toxin A, an *in vivo* toxin neutralization model was developed using Golden Syrian hamsters. This model was based on published values for the minimum amount of toxin A required to elicit diarrhea (0.08 mg toxin A/Kg body wt.) and death (0.16 mg toxin A/Kg body wt.) in hamsters when administered orally (Lyerly *et al.* Infect. Immun., 47:349-352 (1985).

30

For the study, four separate experimental groups were used, with each group consisting of 7 female Golden Syrian hamsters (Charles River), approx, three and one-half weeks old.

weighing approx. 50 gms each. The animals were housed as groups of 3 and 4, and were offered food and water *ad libitum* through the entire length of the study.

For each animal, a mixture containing either 10µg of toxin A (0.2 mg/Kg) or 30µg of toxin A (0.6 mg/Kg) (C. difficile toxin A was obtained from Tech Lab and 1 ml of either the anti-recombinant toxin A IgY or pre-immune IgY (from section (a) above) was prepared. These mixtures were incubated at 37°C for 60 min, and were then administered to the animals by the oral route. The animals were then observed for the onset of diarrhea and death for a period of 24 hrs. following the administration of the toxin A+IgY mixtures, at the end of which time, the following results were tabulated and shown in Table 17:

10

- 5

TABLE 17
Study Outcome At 24 Hours

Experimental Group	Study Outcome at 24 Hours		
	Healthy!	Diarrhea ²	Dead
10 µg Toxin A - Antitoxin Against Interval 6	7	0	0
30 μg Toxin A - Antitoxin Against Interval 6	7	0	
10 μg Toxin A · Pre-Immune Serum	0	5	
30 ng Toxin A · Pre-Immune	0		

15

Animals remained healthy through the entire 24 hour study period.

Animals developed diarrhea, but did not die.

Animals developed diarrhea, and subsequently died.

20

Pretreatment of toxin A at both doses tested, using the anti-recombinant toxin A IgY, prevented all overt symptoms of disease in hamsters. Therefore, pretreatment of C difficile toxin A, using the anti-recombinant toxin A IgY, neutralized the *in vivo* enterotoxin activity of the toxin A. In contrast, all animals from the two groups which received toxin A which had been pretreated using pre-immune IgY developed disease symptoms which ranged from diarrhea to death. The diarrhea which developed in the 5 animals which did not die in each of the two pre-immune groups, spontaneously resolved by the end of the 24 hr. study period.

c) Histologic Evaluation Of Hamster Ccca

30

25

In order to further assess the ability of anti-recombinant toxin A IgY to protect hamsters from the enterotoxin activity of toxin A, histologic evaluations were performed on the ceca of hamsters from the study described in section (b) above.

Three groups of animals were sacrificed in order to prepare histological specimens. The first group consisted of a single representative animal taken from each of the 4 groups of

surviving hamsters at the conclusion of the study described in section (b) above. These animals represented the 24 hr. timepoint of the study.

5

10

15

20

25

30

The second group consisted of two animals which were not part of the study described above, but were separately treated with the same toxin A + pre-immune IgY mixtures as described for the animals in section (b) above. Both of these hamsters developed diarrhea, and were sacrificed 8 hrs. after the time of administration of the toxin A + pre-immune IgY mixtures. At the time of sacrifice, both animals were presenting symptoms of diarrhea. These animals represented the acute phase of the study.

The final group consisted of a single untreated hamster from the same shipment of animals as those used for the two previous groups. This animal served as the normal control.

Samples of cecal tissue were removed from the 7 animals described above, and were fixed overnight at 4°C using 10% buffered formalin. The fixed tissues were paraffinembedded, sectioned, and mounted on glass microscope slides. The tissue sections were then stained using hematoxylin and eosin (II and E stain), and were examined by light microscopy.

The tissues obtained from the two 24 hr. animals which received mixtures containing either $10\mu g$ or $30\mu g$ of toxin A and anti-recombinant toxin A IgY were indistinguishable from the normal control, both in terms of gross pathology, as well as at the microscopic level. These observations provide further evidence for the ability of anti-recombinant toxin A IgY to effectively neutralize the *in vivo* enterotoxin activity of *C. difficile* toxin A, and thus its ability to prevent acute or lasting toxin A-induced pathology.

In contrast, the tissues from the two 24 hr. animals which received the toxin A + preimmune IgY mixtures demonstrated significant pathology. In both of these groups, the mucosal layer was observed to be less organized than in the normal control tissue. The cytoplasm of the epithelial cells had a vacuolated appearance, and gaps were present between the epithelium and the underlying cell layers. The lamina propria was largely absent. Intestinal villi and crypts were significantly diminished, and appeared to have been overgrown by a planar layer of epithelial cells and fibroblasts. Therefore, although these animals overtly appeared to recover from the acute symptoms of toxin A intoxication, lasting pathologic alterations to the cecal mucosa had occurred.

The tissues obtained from the two acute animals which received mixtures of toxin A and pre-immune IgY demonstrated the most significant pathology. At the gross pathological level, both animals were observed to have severely distended ecca which were filled with watery, diarrhea-like material. At the microscopic level, the animal that was given the

mixture containing 10µg of toxin A and pre-immune IgY was found to have a mucosal layer which had a ragged, damaged appearance, and a disorganized, compacted quality. The crypts were largely absent, and numerous breaks in the epithelium had occurred. There was also an influx of erythrocytes into spaces between the epithelial layer and the underlying tissue. The animal which had received the mixture containing 30µg of toxin A and pre-immune IgY demonstrated the most severe pathology. The cecal tissue of this animal had an appearance very similar to that observed in animals which had died from C. difficile disease. Widespread destruction of the mucosa was noted, and the epithelial layer had sloughed. Hemorrhagic areas containing large numbers of erythrocytes were very prevalent. All semblance of normal tissue architecture was absent from this specimen. In terms of the presentation of pathologic events, this in vivo hamster model of toxin A-intoxication correlates very closely with the pathologic consequences of C. difficile disease in hamsters. The results presented in this Example demonstrate that while anti-recombinant toxin A (Interval 6) IgY is capable of only partially neutralizing the cytotoxic activity of C. difficile toxin A, the same antibody effectively neutralizes 100% of the in vivo enterotoxin activity of the toxin. While it is not intended that this invention be limited to this mechanism, this may be due to the cytotoxicity and enterotoxicity of C. difficile Toxin A as two separate and distinct biological functions.

.5

10

15

20

25

30

EXAMPLE 15

In Vivo Neutralization Of C. Difficile Toxin A By
Antibodies Against Recombinant Toxin A Polypeptides

The ability of avian antibodies directed against the recombinant *C. difficile* toxin A fragment 1870-2680 (as expressed by pMA1870-2680; see Example 13) to neutralize the enterotoxic activity of toxin A was demonstrated in Example 14. The ability of avian antibodies (IgYs) directed against other recombinant toxin A epitopes to neutralize native toxin A *in vivo* was next evaluated. This example involved: (a) the preparation of IgYs against recombinant toxin A polypeptides: (b) *in vivo* protection of hamsters against toxin A by treatment with anti-recombinant toxin A IgYs and (c) quantification of specific antibody concentration in CTA and Interval 6 IgY PEG preparations.

The nucleotide sequence of the coding region of the entire toxin A protein is listed in SEQ ID NO:5. The amino acid sequence of the entire toxin A protein is listed in SEQ ID NO:6. The amino acid sequence consisting of amino acid residues 1870 through 2680 of

toxin A is listed in SEQ ID NO:7. The amino acid sequence consisting of amino acid residues 1870 through 1960 of toxin A is listed in SEQ ID NO:8.

a) Preparation Of IgY's Against Recombinant Toxin A Polypeptides

5

10

15

20

25

30

Eggs were collected from Leghorn hens which have been immunized with recombinant C. difficile toxin A polypeptide fragments encompassing the entire toxin A protein. The polypeptide fragments used as immunogens were: 1) pMA 1870-2680 (Interval 6), 2) pPA 1100-1450 (Interval 4), and 3) a mixture of fragments consisting of pMA 30-300 (Interval 1), pMA 300-660 (Interval 2), pMA 660-1100 (Interval 3) and pMA 1450-1870 (Interval 5). This mixture of immunogens is referred to as Interval 1235. The location of each interval within the toxin A molecule is shown in Figure 15A. In Figure 15A, the following abbreviations are used: pP refers to the pET23 vector (New England BioLabs): pM refers to the pMAL. PM-c vector (New England BioLabs): A refers to toxin A: the numbers refer to the amino acid interval expressed in the clone. (For example, the designation pMA30-300 indicates that the recombinant clone encodes amino acids 30-300 of toxin A and the vector used was pMAL. PM-c).

The recombinant proteins were generated as described in Example 11. The IgYs were extracted and solubilized in 0.1M carbonate buffer pH 9.5 for oral administration as described in Example 14(a). The IgY reactivities against each individual recombinant interval was evaluated by ELISA as described in Example 13(c).

b) In Vivo Protection Of Hamsters Against Toxin A By Treatment With Anti-Recombinant Toxin A Antibodies

The ability of antibodies raised against recombinant toxin Λ polypeptides to provide *in vivo* protection against the enterotoxic activity of toxin A was examined in the hamster model system. This assay was performed as described in Example 14(b). Briefly, for each 40-50 gram female Golden Syrian hamster (Charles River), 1 ml of IgY 4X (*i.e.*, resuspended in 1/4 of the original yolk volume) PEG prep against Interval 6, Interval 4 or Interval 1235 was mixed with 30 μg (LD₁₀₀ oral dose) of *C. difficile* toxin Λ (Tech Lab). Preimmune IgY mixed with toxin A served as a negative control. Antibodies raised against *C. difficile* toxoid Λ (Example 8) mixed with toxin Λ (CTΛ) served as a positive control. The mixture was incubated for 1 hour at 37°C then orally administered to lightly etherized hamsters using an

18G feeding needle. The animals were then observed for the onset of diarrhea and death for a period of approximately 24 hours. The results are shown in Table 18.

TABLE 18
Study Outcome After 24 Hours

Treatment group	Healthy!	Diarrhea ²	Dead ¹
Preimmune	0	0	7
СТА	5	0	0
Interval 6	6	ı	0
Interval 4	0	ı	6
Interval 1235	0	0	7

10

. 5

Animal shows no sign of illness.

Animal developed diarrhea, but did not die.

Animal developed diarrhea and died.

15

Pre-treatment of toxin A with IgYs against Interval 6 prevented diarrhea in 6 of 7 hamsters and completely prevented death in all 7. In contrast, as with preimmune IgY, IgYs against Interval 4 and Interval 1235 had no effect on the onset of diarrhea and death in the hamsters.

20

25

c) Quantification Of Specific Antibody Concentration In CTA And Interval 6 IgY PEG Preparations

To determine the purity of IgY PEG preparations, an aliquot of a pMA1870-2680 (Interval 6) IgY PEG preparation was chromatographed using HPLC and a KW-803 sizing column (Shodex). The resulting profile of absorbance at 280 nm is shown in Figure 16. The single large peak corresponds to the predicted MW of IgY. Integration of the area under the single large peak showed that greater than 95% of the protein eluted from the column was present in this single peak. This result demonstrated that the majority (>95%) of the material absorbing at 280 nm in the PEG preparation corresponds to IgY. Therefore, absorbance at 280 nm can be used to determine the total antibody concentration in PEG preparations.

30

35

To determine the concentration of Interval 6-specific antibodies (expressed as percent of total antibody) within the CTA and pMA1870-2680 (Interval 6) PEG preparations, defined quantities of these antibody preparations were affinity purified on a pPA1870-2680(II) (shown schematically in Figure 15B) affinity column and the specific antibodies were quantified. In Figure 15B the following abbreviations are used: pP refers to the pET23 vector (New England BioLabs): pM refers to the pMAL FM-c vector (New England BioLabs): pG refers to the pGEX

vector (Pharmacia): pB refers to the PinPoint^{IM} Xa vector (Promega): A refers to toxin A: the numbers refer to the amino acid interval expressed in the clone. The solid black ovals represent the MBP: the hatched ovals represent glutathione S-transferase: the hatched circles represent the biotin tag: and HHH represents the poly-histidine tag.

5

An affinity column containing recombinant toxin A repeat protein was made as follows. Four ml of PBS-washed Actigel resin (Sterogene) was coupled with 5-10 mg of pPA1870-2680 inclusion body protein [prepared as described in Example (17) and dialyzed into PBS] in a 15 ml tube (Falcon) containing 1/10 final volume Ald-coupling solution (1 M sodium cyanoborohydride). Aliquots of the supernatant from the coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based upon protein band intensities, greater than 6 mg of recombinant protein was coupled to the resin. The resin was poured into a 10 ml column (BioRad), washed extensively with PBS, pre-cluted with 4 M guanidine-HCI (in 10 mM Tris-HCI, pH 8.0; 0.005% thimerosal) and reequilibrated with PBS. The column was stored at 4°C.

15

20

10

Aliquots of a pMA1870-2680 (Interval 6) or a CTA IgY polyclonal antibody preparation (PEG prep) were affinity purified on the above affinity column as follows. The column was attached to an UV monitor (ISCO) and washed with PBS. For pMA1870-2680 IgY purification, a 2X PEG prep (filter sterilized using a 0.45 μ filter; approximately 500 mg total IgY) was applied. The column was washed with PBS until the baseline was reestablished (the column flow-through was saved), washed with BBSTween to elute nonspecifically binding antibodies and re-equilibrated with PBS. Bound antibody was eluted from the column in 4 M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0; 0.005% thimerosal). The entire elution peak was collected in a 15 ml tube (Falcon). The column was reequilibrated and the column cluate was re-chromatographed as described above. The antibody preparation was quantified by UV absorbance (the elution buffer was used to zero the spectrophotometer). Total purified antibody was approximately 9 mg and 1 mg from the first and second chromatography passes, respectively. The low yield from the second pass indicated that most specific antibodies were removed by the first round of chromatography. The estimated percentage of Interval 6 specific antibodies in the pMA1870-2680 PEG prep is approximately 2%.

30

25

The percentage of Interval 6 specific antibodies in the CTA PEG prep was determined (utilizing the same column and methodology described above) to be approximately 0.5% of total IgY.

A 4X PEG prep contains approximately 20 mg/ml IgY. Thus in b) above, approximately 400 μ g specific antibody in the Interval 6 PEG prep neutralized 30 μ g toxin A in vivo.

EXAMPLE 16

In Vivo Treatment Of C. difficile Disease In Hamsters By Recombinant Interval 6 Antibodies

The ability of antibodies directed against recombinant Interval 6 of toxin A to protect hamsters in vivo from C. difficile disease was examined. This example involved: (a) prophylactic treatment of C. difficile disease and (b) therapeutic treatment of C. difficile disease.

a) Prophylactic Treatment Of C. difficile Disease

. 5

10

15

20

25

This experiment was performed as described in Example 9(b). Three groups each consisting of 7 female 100 gram Syrian hamsters (Charles River) were prophylactically treated with either preimmune IgYs, IgYs against native toxin A and B [CTAB: see Example 8 (a) and (b)] or IgYs against Interval 6. IgYs were prepared as 4X PEG preparations as described in Example 9(a).

The animals were orally dosed 3 times daily, roughly at 4 hour intervals, for 12 days with 1 ml antibody preparations diluted in Ensure®. Using estimates of specific antibody concentration from Example 15(c), each dose of the Interval 6 antibody prep contained approximately 400 µg of specific antibody. On day 2 each hamster was predisposed to C. difficile infection by the oral administration of 3.0 mg of Clindamycin-HCl (Sigma) in 1 ml of water. On day 3 the hamsters were orally challenged with 1 ml of C. difficile inoculum strain ATCC 43596 in sterile saline containing approximately 100 organisms. The animals were then observed for the onset of diarrhea and subsequent death during the treatment period. The results are shown in Table 19.

TABLE 19
Lethality After 12 Days Of Treatment

Treatment Group	Number Animals Alive	Number Animals Dead
Preimmune	0	7
СТАВ	6	
Interval 6	7	()

5

10

15

20

25

30

Treatment of hamsters with orally-administered IgYs against Interval 6 successfully protected 7 out of 7 (100%) of the animals from *C. difficile* disease. One of the hamsters in this group presented with diarrhea which subsequently resolved during the course of treatment. As shown previously in Example 9, antibodies to native toxin A and toxin B were highly protective. In this Example, 6 out of 7 animals survived in the CTAB treatment group. All of the hamsters treated with preimmune sera came down with diarrhea and died. The survivors in both the CTAB and Interval 6 groups remained healthy throughout a 12 day post-treatment period. In particular, 6 out of 7 Interval 6-treated hamsters survived at least 2 weeks after termination of treatment which suggests that these antibodies provide a long-lasting cure. These results represent the first demonstration that antibodies generated against a recombinant region of toxin A can prevent CDAD when administered passively to animals. These results also indicate that antibodies raised against Interval 6 alone may be sufficient to protect animals from *C. difficile* disease when administered prophylactically.

Previously others had raised antibodies against toxin A by actively immunizing hamsters against a recombinant polypeptide located within the Interval 6 region [Lyerly, D.M., et al. (1990) Curr. Microbiol. 21:29]. Figure 17 shows schematically the location of the Lyerly, et al. intra-Interval 6 recombinant protein (cloned into the pUC vector) in comparison with the complete Interval 6 construct (pMA1870-2680) used herein to generate neutralizing antibodies directed against toxin A. In Figure 17, the solid black oval represents the MBP which is fused to the toxin A Interval 6 in pMA1870-2680.

The Lyerly, et al. antibodies (intra-Interval 6) were only able to partially protect hamsters against C. difficile infection in terms of survival (4 out of 8 animals survived) and furthermore, these antibodies did not prevent diarrhea in any of the animals. Additionally, animals treated with the intra-Interval 6 antibodies [Lyerly, et al. (1990), supra] died when treatment was removed.

In contrast, the experiment shown above demonstrates that passive administration of anti-Interval 6 antibodies prevented diarrhea in 6 out of 7 animals and completely prevented

death due to CDAD. Furthermore, as discussed above, passive administration of the anti-Interval 6 antibodies provides a long lasting cure (i.e., treatment could be withdrawn without incident).

b) Therapeutic Treatment Of C. difficile Disease: In Vivo Treatment Of An Established C. difficile Infection In Hamsters With Recombinant Interval 6 Antibodies

. 5

10

15

20

25

30

The ability of antibodies against recombinant interval 6 of toxin A to therapeutically treat C. difficile disease was examined. The experiment was performed essentially as described in Example 10(b). Three groups, each containing seven to eight female Golden Syrian hamsters (100 g each: Charles River) were treated with either preimmune IgY, IgYs against native toxin A and toxin B (CTAB) and IgYs against Interval 6. The antibodies were prepared as described above as 4X PEG preparations.

The hamsters were first predisposed to *C. difficile* infection with a 3 mg dose of Clindamycin-HCl (Sigma) administered orally in 1 ml of water. Approximately 24 hrs later, the animals were orally challenged with 1 ml of *C. difficile* strain ATCC 43596 in sterile saline containing approximately 200 organisms. One day after infection, the presence of toxin A and B was determined in the feces of the hamsters using a commercial immunoassay kit (Cytoclone A+B EPA, Cambridge Biotech) to verify establishment of infection. Four members of each group were randomly selected and tested. Feces from an uninfected hamster was tested as a negative control. All infected animals tested positive for the presence of toxin according to the manufacturer's procedure. The initiation of treatment then started approximately 24 hr post-infection.

The animals were dosed daily at roughly 4 hr intervals with 1 ml antibody preparation diluted in Ensure® (Ross Labs). The amount of specific antibodies given per dose (determined by affinity purification) was estimated to be about 400 µg of anti-Interval 6 IgY (for animals in the Interval 6 group) and 100 µg and 70 µg of anti-toxin A (Interval 6-specific) and anti-toxin B (Interval 3-specific; see Example 19), respectively, for the CTAB preparation. The animals were treated for 9 days and then observed for an additional 4 days for the presence of diarrhea and death. The results indicating the number of survivors and the number of dead 4 days post-infection are shown in Table 20.

TABLE 20
In vivo Therapeutic Treatment With Interval 6 Antibodies

Treatment Group	Number Animals Alive	Number Animals Dead
Preimmune	4	ż
CTAB	8	0
Interval 6	8	0

Antibodies directed against both Interval 6 and CTAB successfully prevented death from C. difficile when therapeutically administered 24 hr after infection. This result is significant since many investigators begin therapeutic treatment of hamsters with existing drugs (e.g., vancomycin, phenelfamycins, tiacumicins, etc.) 8 hr post-infection [Swanson, et al. (1991) Antimicrobial Agents and Chemotherapy 35:1108 and (1989) J. Antibiotics 42:94].

Forty-two percent of hamsters treated with preimmune IgY died from CDAD. While the anti-Interval 6 antibodies prevented death in the treated hamsters, they did not eliminate all symptoms of CDAD as 3 animals presented with slight diarrhea. In addition, one CTAB-treated and one preimmune-treated animal also had diarrhea 14 days post-infection. These results indicate that anti-Interval 6 antibodies provide an effective means of therapy for CDAD.

20 EXAMPLE 17

5

10

15

25

30

Induction Of Toxin A Neutralizing Antibodies Requires Soluble Interval 6 Protein

As shown in Examples 11(d) and 15, expression of recombinant proteins in E, coli may result in the production of either soluble or insoluble protein. If insoluble protein is produced, the recombinant protein is solubilized prior to immunization of animals. To determine whether, one or both of the soluble or insoluble recombinant proteins could be used to generate neutralizing antibodies to toxin A, the following experiment was performed. This example involved a) expression of the toxin A repeats and subfragments of these repeats in E, coli using a variety of expression vectors; b) identification of recombinant toxin A repeats and sub-regions to which neutralizing antibodies bind; and c) determination of the neutralization ability of antibodies raised against soluble and insoluble toxin A repeat immunogen.

a) Expression Of The Toxin A Repeats And Subfragments Of These Repeats In E. coli Using A Variety Of Expression Vect rs

. 2

10

15

20

25

30

The Interval 6 immunogen utilized in Examples 15 and 16 was the pMA1870-2680 protein, in which the toxin A repeats are expressed as a soluble fusion protein with the MBP (described in Example 11). Interestingly, expression of this region (from the *Spel* site to the end of the repeats, see Figure 15B) in three other expression constructs, as either native (pPA1870-2680), poly-His tagged [pPA1870-2680 (H)] or biotin-tagged (pBA1870-2680) proteins resulted in completely insoluble protein upon induction of the bacterial host (see Figure 15B). The host strain BL21 (Novagen) was used for expression of pBA1870-2680 and host strain BL21(DE3) (Novagen) was used for expression of pPA1870-2680 and pPA1870-2680(H). These insoluble proteins accumulated to high levels in inclusion bodies. Expression of recombinant plasmids in *E. coli* host cells grown in 2X YT medium was performed as described [Williams, *et al.* (1995), *supra*].

As summarized in Figure 15B, expression of fragments of the toxin A repeats (as either N-terminal *Spel-Eco*RI fragments, or C-terminal *Eco*RI-end fragments) also yielded high levels of insoluble protein using pGEX (pGA1870-2190). PinPointTM-Xa (pBA1870-2190 and pBA2250-2680) and pET expression systems (pPA1870-2190). The pGEX and pET expression systems are described in Example 11. The PinPointTM-Xa expression system drives the expression of fusion proteins in *E. coli*. Fusion proteins from PinPointTM-Xa vectors contain a biotin tag at the amino-terminal end and can be affinity purified SoftLinkTM Soft Release avidin resin (Promega) under mild denaturing conditions (5 mM biotin).

The solubility of expressed proteins from the pPG1870-2190 and pPA1870-2190 expression constructs was determined after induction of recombinant protein expression under conditions reported to enhance protein solubility [These conditions comprise growth of the host at reduced temperature (30°C) and the utilization of high (1 mM IPTG) or low (0.1 mM IPTG) concentrations of inducer [Williams et al. (1995), supra]. All expressed recombinant toxin A protein was insoluble under these conditions. Thus, expression of these fragments of the toxin A repeats in pET and pGEX expression vectors results in the production of insoluble recombinant protein even when the host cells are grown at reduced temperature and using lower concentrations of the inducer. Although expression of these fragments in pMal vectors yielded affinity purifiable soluble fusion protein, the protein was either predominantly insoluble (pMA1870-2190) or unstable (pMA2250-2650). Attempts to solubilize expressed

protein from the pMA1870-2190 expression construct using reduced temperature or lower inducer concentration (as described above) did not improve fusion protein solubility.

Collectively, these results demonstrate that expression of the toxin A repeat region in *E. coli* results in the production of insoluble recombinant protein, when expressed as either large (aa 1870-2680) or small (aa 1870-2190 or aa 2250-2680) fragments, in a variety of expression vectors (native or poly-his tagged pET, pGEX or PinPoint^{1M}- Xa vectors), utilizing growth conditions shown to enhance protein solubility. The exception to this rule were fusions with the MBP, which enhanced protein solubility, either partially (pMA1870-2190) or fully (pMA1870-2680).

10

15

20

25

30

5

b) Identification Of Recombinant Toxin A Repeats And Sub-Regions To Which Neutralizing Antibodies Bind

Toxin A repeat regions to which neutralizing antibodies bind were identified by utilizing recombinant toxin A repeat region proteins expressed as soluble or insoluble proteins to deplete protective antibodies from a polyclonal pool of antibodies against native *C. difficile* toxin A. An *in vivo* assay was developed to evaluate proteins for the ability to bind neutralizing antibodies.

The rational for this assay is as follows. Recombinant proteins were first pre-mixed with antibodies against native toxin A (CTA antibody; generated in Example 8) and allowed to react. Subsequently, C. difficile toxin A was added at a concentration lethal to hamsters and the mixture was administered to hamsters via IP injection. If the recombinant protein contains neutralizing epitopes, the CTA antibodies would lose their ability to bind toxin A resulting in diarrhea and/or death of the hamsters.

The assay was performed as follows. The lethal dose of toxin A when delivered orally to nine 40 to 50 g Golden Syrian hamsters (Sasco) was determined to be 10 to 30 μg. The PEG-purified CTA antibody preparation was diluted to 0.5X concentration (i.e., the antibodies were diluted at twice the original yolk volume) in 0.1 M carbonate buffer, pH 9.5. The antibodies were diluted in carbonate buffer to protect them from acid degradation in the stomach. The concentration of 0.5X was used because it was found to be the lowest effective concentration against toxin A. The concentration of Interval 6-specific antibodies in the 0.5X CTA prep was estimated to be 10-15 μg/ml (estimated using the method described in Example 15).

The inclusion body preparation [insoluble Interval 6 protein: pPA1870-2680(H)] and the soluble Interval 6 protein [pMA1870-2680; see Figure 15] were both compared for their ability to bind to neutralizing antibodies against *C. difficile* toxin A (CTA). Specifically, I to 2 mg of recombinant protein was mixed with 5 ml of a 0.5X CTA antibody prep (estimated to contain 60-70 µg of Interval 6-specific antibody). After incubation for 1 hr at 37°C, CTA (Tech Lab) at a final concentration of 30 µg/ml was added and incubated for another 1 hr at 37°C. One ml of this mixture containing 30 µg of toxin A (and 10-15 µg of Interval 6-specific antibody) was administered orally to 40-50 g Golden Syrian hamsters (Sasco). Recombinant proteins that result in the loss of neutralizing capacity of the CTA antibody would indicate that those proteins contain neutralizing epitopes. Preimmune and CTA antibodies (both at 0.5X) without the addition of any recombinant protein served as negative and positive controls, respectively.

Two other inclusion body preparations, both expressed as insoluble products in the pET vector, were tested; one containing a different insert (toxin B fragment) other than Interval 6 called pPB1850-2070 (see Figure 18) which serves as a control for insoluble Interval 6, the other was a truncated version of the Interval 6 region called pPA1870-2190 (see Figure 15B). The results of this experiment are shown in Table 21.

TABLE 21

Binding Of Neutralizing Antibodies By Soluble Interval 6 Protein Study Outcome After 24 Hours

Treatment Group!	Healthy ²	Diarrhea ¹	Dead'
Preimmune Ab	0	3	2
CTA Ab	4	I	()
CTA Ab - Int 6 (soluble)	1	2	· · · · · · · · · · · · · · · · · · ·
CTA Ab - Int 6 (insoluble)	5	0	0
CTA Ab + pPB1850-2070	5	0	n
CTA Ab + pPA1870-2190	5	0	

C. difficile toxin A (CTA) was added to each group.

Animals showed no signs of illness.

. 5

10

15

20

25

30

35

Animals developed diarrhea but did not die.

Animals developed diarrhea and died.

Preimmune antibody was ineffective against toxin A, while anti-CTA antibodies at a dilute 0.5X concentration almost completely protected the hamsters against the enterotoxic effects of CTA. The addition of recombinant proteins pPB1850-2070 or pPA1870-2190 to the anti-CTA antibody had no effect upon its protective ability, indicating that these recombinant proteins do not bind to neutralizing antibodies. On the other hand, recombinant

Interval 6 protein was able to bind to neutralizing anti-CTA antibodies and neutralized the *in vivo* protective effect of the anti-CTA antibodies. Four out of five animals in the group treated with anti-CTA antibodies mixed with soluble Interval 6 protein exhibited toxin associated toxicity (diarrhea and death). Moreover, the results showed that Interval 6 protein must be expressed as a soluble product in order for it to bind to neutralizing anti-CTA antibodies since the addition of insoluble Interval 6 protein had no effect on the neutralizing capacity of the CTA antibody prep.

c) Determination Of Neutralization Ability Of Antibodies Raised Against Soluble And Insoluble Toxin A Repeat Immunogen

5

10

15

20

25

30

To determine if neutralizing antibodies are induced against solubilized inclusion bodies, insoluble toxin A repeat protein was solubilized and specific antibodies were raised in chickens. Insoluble pPA1870-2680 protein was solubilized using the method described in Williams *et al.* (1995), *supra*. Briefly, induced cultures (500 ml) were pelleted by centrifugation at 3,000 X g for 10 min at 4°C. The cell pellets were resuspended thoroughly in 10 ml of inclusion body sonication buffer (25 mM HEPES pH 7.7, 100 mM KCl, 12.5 mM MgCl., 20% glycerol, 0.1% (v/v) Nonidet P-40. 1 mM DTT). The suspension was transferred to a 30 ml non-glass centrifuge tube. Five hundred µl of 10 mg/ml lysozyme was added and the tubes were incubated on ice for 30 min. The suspension was then frozen at -70°C for at least 1 hr. The suspension was thawed rapidly in a water bath at room temperature and then placed on ice. The suspension was then sonicated using at least eight 15 sec bursts of the microprobe (Branson Sonicator Model No. 450) with intermittent cooling on ice.

The sonicated suspension was transferred to a 35 ml Oakridge tube and centrifuged at 6.000 X g for 10 min at 4°C to pellet the inclusion bodies. The pellet was washed 2 times by pipetting or vortexing in fresh, ice-cold RIPA buffer [0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate in TBS (25 mM Tris-Cl pH 7.5, 150 mM NaCl)]. The inclusion bodies were recentrifuged after each wash. The inclusion bodies were dried and transferred using a small metal spatula to a 15 ml tube (Falcon). One ml of 10% SDS was added and the pellet was solubilized by gently pipetting the solution up and down using a 1 ml micropipettor. The solubilization was facilitated by heating the sample to 95°C when necessary.

Once the inclusion bodies were in solution, the samples were diluted with 9 volumes of PBS. The protein solutions were dialyzed overnight against a 100-fold volume of PBS

containing 0.05% SDS at room temperature. The dialysis buffer was then changed to PBS containing 0.01% SDS and the samples were dialyzed for several hours to overnight at room temperature. The samples were stored at 4°C until used. Prior to further use, the samples were warmed to room temperature to allow any precipitated SDS to go back into solution.

The inclusion body preparation was used to immunize hens. The protein was dialyzed into PBS and emulsified with approximately equal volumes of CFA for the initial immunization or IFA for subsequent booster immunizations. On day zero, for each of the recombinant recombinant preparations, two egg laying white Leghorn hens were each injected at multiple sites (IM and SC) with 1 ml of recombinant protein-adjuvant mixture containing approximately 0.5-1.5 mg of recombinant protein. Booster immunizations of 1.0 mg were given of days 14 and day 28. Eggs were collected on day 32 and the antibody isolated using PEG as described in Example 14(a). High titers of toxin A specific antibodies were present (as assayed by ELISA, using the method described in Example 13). Titers were determined for both antibodies against recombinant polypeptides pPA1870-2680 and pMA1870-2680 and were found to be comparable at > 1:62,500.

Antibodies against soluble Interval 6 (pMA1870-2680) and insoluble Interval 6 [(inclusion body), pPA1870-2680] were tested for neutralizing ability against toxin A using the *in vivo* assay described in Example 15(b). Preimmune antibodies and antibodies against toxin A (CTA) served as negative and positive controls, respectively. The results are shown in Table 22.

TABLE 22
Neutralization Of Toxin A By Antibodies Against Soluble Interval 6 Protein Study Outcome After 24 Hours

Antibody Treatment Group	Healthy 1	Diarrhea ²	Dead
Preimmune	1	0	-4
СТА	5	0	<u>(</u>)
Interval 6 (Soluble)	5	0	
Interval 6 (Insoluble)	θ	7	

Animals showed no sign of illness.

Animal developed diarrhea but did not die.

Animal developed diarrhea and died.

400 րջ տե

. 5

10

15

20

25

30

35

Antibodies raised against native toxin A were protective while preimmune antibodies had little effect. As found using the *in vitro* CHO assay [described in Example 8(d)] where antibodies raised against the soluble Interval 6 could partially neutralize the effects of toxin A, here they were able to completely neutralize toxin A *in vivo*. In contrast, the antibodies

raised against the insoluble Interval 6 was unable to neutralize the effects of toxin A in vivo as shown above (Table 22) and in vitro as shown in the CHO assay [described in Example 8(d)].

These results demonstrate that soluble toxin A repeat immunogen is necessary to induce the production of neutralizing antibodies in chickens, and that the generation of such soluble immunogen is obtained only with a specific expression vector (pMal) containing the toxin A region spanning as 1870-2680. That is to say, insoluble protein that is subsequently solubilized does not result in a toxin A antigen that will elicit a neutralizing antibody.

10

15

5

EXAMPLE 18

Cloning And Expression Of The C. difficile Toxin B Gene

The toxin B gene has been cloned and sequenced: the amino acid sequence deduced from the cloned nucleotide sequence predicts a MW of 269.7 kD for toxin B [Barroso et al., Nucl. Acids Res. 18:4004 (1990)]. The nucleotide sequence of the coding region of the entire toxin B gene is listed in SEQ ID NO:9. The amino acid sequence of the entire toxin B protein is listed in SEQ ID NO:10. The amino acid sequence consisting of amino acid residues 1850 through 2360 of toxin B is listed in SEQ ID NO:11. The amino acid sequence consisting of amino acid residues 1750 through 2360 of toxin B is listed in SEQ ID NO:12.

20

25

Given the expense and difficulty of isolating native toxin B protein, it would be advantageous to use simple and inexpensive procaryotic expression systems to produce and purify high levels of recombinant toxin B protein for immunization purposes. Ideally, the isolated recombinant protein would be soluble in order to preserve native antigenicity, since solubilized inclusion body proteins often do not fold into native conformations. Indeed as shown in Example 17, neutralizing antibodies against recombinant toxin A were only obtained when soluble recombinant toxin A polypeptides were used as the immunogen. To allow ease of purification, the recombinant protein should be expressed to levels greater than 1 mg/liter of *E. coli* culture.

30

To determine whether high levels of recombinant toxin B protein could be produced in E. coli. fragments of the toxin B gene were cloned into various prokaryotic expression vectors, and assessed for the ability to express recombinant toxin B protein in E. coli. This Example involved (a) cloning of the toxin B gene and (b) expression of the toxin B gene in E. coli.

a) Cloning Of The Toxin B Gene

- 5

10

15

20

25

30

The toxin B gene was cloned using PCR amplification from *C. difficile* genomic DNA. Initially, the gene was cloned in two overlapping fragments, using primer pairs P5/P6 and P7/P8. The location of these primers along the toxin B gene is shown schematically in Figure 18. The sequence of each of these primers is: P5: 5 TAGAAAAAATGGCAAATGT 3 (SEQ ID NO:11): P6: 5 TTTCATCTTGTA GAGTCAAAG 3 (SEQ ID NO:12):

P7: 5' GATGCCACAAGATGATTTAGTG 3' (SEQ ID NO:13); and P8: 5' CTAATTGAGCTGTATCAGGATC 3' (SEQ ID NO:14).

Figure 18 also shows the location of the following primers along the toxin B gene: P9 which consists of the sequence 5' CGGAATTCCTAGAAAAAATGGCAA ATG 3' (SEQ ID NO:15): P10 which consists of the sequence 5' GCTCTAGAATGA CCATAAGCTAGCCA 3' (SEQ ID NO:16): P11 which consists of the sequence 5' CGGAATTCGAGTTGGTAGAAAGGTGGA 3' (SEQ ID NO:17): P13 which consists of the sequence 5' CGGAATTCGGTTATTATCTTAAGGATG 3' (SEQ ID NO:18): and P14 which consists of the sequence 5' CGGAATTCTTGATAACTGGAT TTGTGAC 3' (SEQ ID NO:19). The amino acid sequence consisting of amino acid residues 1852 through 2362 of toxin B is listed in SEQ ID NO:20. The amino acid sequence consisting of amino acid residues 1755 through 2362 of toxin B is listed in SEQ ID NO:21.

Collection (ATCC 43255) and grown under anaerobic conditions in brain-heart infusion medium (Becton Dickinson). High molecular-weight C. difficile DNA was isolated essentially as described [Wren and Tabaqchali (1987) J. Clin. Microbiol., 25:2402], except 1) 100 μg/ml proteinase K in 0.5% SDS was used to disrupt the bacteria and 2) cetytrimethylammonium bromide (CTAB) precipitation [as described by Ausubel et al., Eds., Current Protocols in Molecular Biology, Vol. 2 (1989) Current Protocols] was used to remove carbohydrates from the cleared lysate. Briefly, after disruption of the bacteria with proteinase K and SDS, the solution is adjusted to approximately 0.7 M NaCl by the addition of a 1/7 volume of 5M NaCl. A 1/10 volume of CTAB/NaCl (10% CTAB in 0.7 M NaCl) solution was added and the solution was mixed thoroughly and incubated 10 min at 65°C. An equal volume of chloroform/isoamyl alcohol (24:1) was added and the phases were thoroughly mixed. The organic and aqueous phases were separated by centrifugation in a microfuge for 5 min. The aqueous supernatant was removed and extracted with phenol/chloroform/ isoamyl alcohol (25:24:1). The phases were separated by centrifugation in a microfuge for 5 min. The

supernatant was transferred to a fresh tube and the DNA was precipitated with isopropanol. The DNA precipitate was pelleted by brief centrifugation in a microfuge. The DNA pellet was washed with 70% ethanol to remove residual CTAB. The DNA pellet was then dried and redissolved in TE buffer (10 mM Tris-HCl pH8.0. 1 mM EDTA). The integrity and yield of genomic DNA was assessed by comparison with a serial dilution of uncut lambda DNA after electrophoresis on an agarose gel.

5

10

15

20

25

30

Toxin B fragments were cloned by PCR utilizing a proofreading thermostable DNA polymerase [native *Pfu* polymerase (Stratagene)]. The high fidelity of this polymerase reduces the mutation problems associated with amplification by error prone polymerases (*e.g.*, *Taq* polymerase). PCR amplification was performed using the PCR primer pairs P5 (SEQ ID NO:11) with P6 (SEQ ID NO:12) and P7 (SEQ ID NO:13) with P8 (SEQ ID NO:14) in 50 μl reactions containing 10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM MgCl, 200 μM of each dNTP, 0.2 μM each primer, and 50 ng *C. difficile* genomic DNA. Reactions were overlaid with 100 μl mineral oil, heated to 94°C for 4 min, 0.5μl native *Pfu* polymerase (Stratagene) was added, and the reactions were cycled 30 times at 94°C for 1 min, 50°C for 1 min, 72°C (2 min for each kb of sequence to be amplified), followed by 10 min at 72°C. Duplicate reactions were pooled, chloroform extracted, and ethanol precipitated. After washing in 70% ethanol, the pellets were resuspended in 50 μl TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA).

The P5/P6 amplification product was cloned into pUC19 as outlined below. 10µl aliquots of DNA were gel purified using the Prep-a-Gene kit (BioRad), and ligated to *Smal* restricted pUC19 vector. Recombinant clones were isolated and confirmed by restriction digestion using standard recombinant molecular biology techniques (Sambrook *et al.*, 1989). Inserts from two independent isolates were identified in which the toxin B insert was oriented such that the vector *BamHI* and *SacI* sites were 5° and 3° oriented, respectively (pUCB10-1530). The insert-containing *BamHI/SacI* fragment was cloned into similarly cut pET23a-c vector DNA, and protein expression was induced in small scale cultures (5 ml) of identified clones.

Total protein extracts were isolated, resolved on SDS-PAGE gels, and toxin B protein identified by Western analysis utilizing a goat anti-toxin B affinity purified antibody (Tech Lab). Procedures for protein induction, SDS-PAGE, and Western blot analysis were performed as described in Williams *et al.* (1995), *supra*. In brief, 5 ml cultures of bacteria grown in 2XYT containing 100 μg/ml ampicillin containing the appropriate recombinant clone

were induced to express recombinant protein by addition of IPTG to ImM. The E. coli hosts used were: BL21(DE3) or BL21(DE3)LysS (Novagen) for pET plasmids.

Cultures were induced by the addition of IPTG to a final concentration of 1.0 mM when the cell density reached 0.5 OD₆₀₀, and induced protein was allowed to accumulate for two hrs after induction. Protein samples were prepared by pelleting 1 ml aliquots of bacteria by centrifugation (1 min in microfuge), and resuspension of the pelleted bacteria in 150 μl of 2X SDS-PAGE sample buffer (0.125 mM Tris-HCl pH 6.8, 2 mM EDTA, 6% SDS, 20% glycerol, 0.025% bromophenol blue; β-mercaptoethanol is added to 5% before use). The samples were heated to 95°C for 5 min, then cooled and 5 or 10 μls loaded on 7.5% SDS-PAGE gels. High molecular weight protein markers (BioRad) were also loaded, to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining the gels with Coomassie Blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein. The MW of induced toxin B reactive protein allowed the integrity of the toxin B reading frame to be determined.

The pET23b recombinant (pPB10-1530) expressed high MW recombinant toxin B reactive protein, consistent with predicted values. This confirmed that frame terminating errors had not occurred during PCR amplification. A pET23b expression clone containing the 10-1750aa interval of the toxin B gene was constructed, by fusion of the EcoRV-Spel fragment of the P7/P8 amplification product to the P5-EcoRV interval of the P5/P6 amplification product (see Figure 18) in pPB10-1530. The integrity of this clone (pPB10-1750) was confirmed by restriction mapping, and Western blot detection of expressed recombinant toxin B protein. Levels of induced protein from both pPB10-1530 and pPB10-1750 were too low to facilitate purification of usable amounts of recombinant toxin B protein. The remaining 1750-2360 aa interval was directly cloned into pMAL or pET expression vectors from the P7/P8 amplification product as described below.

b) Expression Of The Toxin B Gene

• 5

10

15

20

25

30

i) Overview Of Expression Methodologies

Fragments of the toxin B gene were expressed as either native or fusion proteins in E. coli. Native proteins were expressed in either the pET23a-c or pET16b expression vectors (Novagen). The pET23 vectors contain an extensive polylinker sequence in all three reading frames (a-c vectors) followed by a C-terminal poly-histidine repeat. The pET16b vector

contains a N-terminal poly-histidine sequence immediately 5° to a small polylinker. The poly-histidine sequence binds to Ni-Chelate columns and allows affinity purification of tagged target proteins [Williams et al. (1995), supra]. These affinity tags are small (10 aa for pET16b. 6 aa for pET23) allowing the expression and affinity purification of native proteins with only limited amounts of foreign sequences.

5

10

15

20

25

30

An N-terminal histidine-tagged derivative of pET16b containing an extensive cloning cassette was constructed to facilitate cloning of N-terminal poly-histidine tagged toxin B expressing constructs. This was accomplished by replacement of the promoter region of the pET23a and b vectors with that of the pET16b expression vector. Each vector was restricted with Bg/II and NdeI, and the reactions resolved on a 1.2 % agarose gel. The pET16b promoter region (contained in a 200 bp Bg/II-NdeI fragment) and the promoter-less pET23 a or b vectors were cut from the gel, mixed and Prep-A-Gene (BioRad) purified. The eluted DNA was ligated, and transformants screened for promoter replacement by NcoI digestion of purified plasmid DNA (the pET16b promoter contains this site, the pET23 promoter does not). These clones (denoted pETHisa or b) contain the pET16b promoter (consisting of a pT7-lac promoter, ribosome binding site and poly-histidine (10aa) sequence) fused at the NdeI site to the extensive pET23 polylinker.

All MBP fusion proteins were constructed and expressed in the pMAL.^{IM}-c or pMAL.^{IM}-p2 vectors (New England Biolabs) in which the protein of interest is expressed as a C-terminal fusion with MBP. All pET plasmids were expressed in either the Bl.21(DE3) or BL21(DE3)LysS expression hosts, while pMal plasmids were expressed in the Bl.21 host.

Large scale (500 mls to 1 liter) cultures of each recombinant were grown in 2X YT broth, induced, and soluble protein fractions were isolated as described [Williams, et al. (1995), *supra*]. The soluble protein extracts were affinity chromatographed to isolate recombinant fusion protein, as described [Williams *et al.*, (1995) *supra*]. In brief, extracts containing tagged pET fusions were chromatographed on a nickel chelate column, and eluted using imidazole salts or low pH (pH 4.0) as described by the distributor (Novagen or Qiagen). Extracts containing soluble pMAL fusion protein were prepared and chromatographed in PBS buffer over an amylose resin (New England Biolabs) column, and eluted with PBS containing 10 mM maltose as described [Williams *et al.* (1995), *supra*].

ii) Overview Of Toxin B Expression

. 5

10

15

20

25

30

In both large expression constructs described in (a) above, only low level (i.e., less than 1 mg/liter of intact or nondegraded recombinant protein) expression of recombinant protein was detected. A number of expression constructs containing smaller fragments of the toxin B gene were then constructed, to determine if small regions of the gene can be expressed to high levels (i.e., greater than 1 mg/liter intact protein) without extensive protein degradation. All were constructed by in frame fusions of convenient toxin B restriction fragments to either the pMAL-c, pET23a-c, pET16b or pETHisa-b expression vectors, or by engineering restriction sites at specific locations using PCR amplification [using the same conditions described in (a) above]. In all cases, clones were verified by restriction mapping, and, where indicated, DNA sequencing.

Protein preparations from induced cultures of each of these constructs were analyzed, by SDS-PAGE, to estimate protein stability (Coomassie Blue staining) and immunoreactivity against anti-toxin B specific antiserum (Western analysis). Higher levels of intact (i.e., nondegraded), full length fusion proteins were observed with the smaller constructs as compared with the larger recombinants, and a series of expression constructs spanning the entire toxin B gene were constructed (Figures 18, 19 and 20 and Table 23).

Constructs that expressed significant levels of recombinant toxin B protein (greater than 1 mg/liter intact recombinant protein) in *E. coli* were identified and a series of these clones that spans the toxin B gene are shown in Figure 19 and summarized in Table 23. These clones were utilized to isolate pure toxin B recombinant protein from the entire toxin B gene. Significant protein yields were obtained from pMAL expression constructs spanning the entire toxin B gene, and yields of full length soluble fusion protein ranged from an estimated 1 mg/liter culture (pMB1100-1530) to greater than 20 mg/liter culture (pMB1750-2360).

Representative purifications of MBP and poly-histidine-tagged toxin B recombinants are shown in Figures 21 and 22. Figure 21 shows a Coomassie Blue stained 7.5% SDS-PAGE gel on which various protein samples extracted from bacteria harboring pMB1850-2360 were electrophoresed. Samples were loaded as follows: Lane 1: protein extracted from uninduced culture: Lane 2: induced culture protein: Lane 3: total protein from induced culture after sonication: Lane 4: soluble protein: and Lane 5: eluted affinity purified protein. Figure 22 depicts the purification of recombinant proteins expressed in bacteria harboring either pPB1850-2360 (Lanes 1-3) or pPB1750-2360 (Lanes 4-6). Samples were loaded as follows: uninduced total protein (Lanes 1 and 4): induced total protein (Lanes 2 and 5): and eluted

affinity purified protein (Lanes 3 and 6). The broad range molecular weight protein markers (BioRad) are shown in Lane 7.

Thus, although high level expression was not attained using large expression constructs from the toxin B gene, usable levels of recombinant protein were obtained by isolating induced protein from a series of smaller pMAL expression constructs that span the entire toxin B gene.

5 .

10

15

20

25

30

These results represent the first demonstration of the feasibility of expressing recombinant toxin B protein to high levels in *E. coli*. As well, expression of small regions of the putative ligand binding domain (repeat region) of toxin B as native protein yielded insoluble protein, while large constructs, or fusions to MBP were soluble (Figure 19), demonstrating that specific methodologies are necessary to produce soluble fusion protein from this interval.

iii) Clone Construction And Expression Details

A portion of the toxin B gene containing the toxin B repeat region [amino acid residues 1852-2362 of toxin B (SEQ ID NO:20)] was isolated by PCR amplification of this interval of the toxin B gene from C. difficile genomic DNA. The sequence, and location within the toxin B gene, of the two PCR primers [P7 (SEQ ID NO:13) and P8 (SEQ ID NO:14)] used to amplify this region are shown in Figure 18.

DNA from the PCR amplification was purified by chloroform extraction and ethanol precipitation as described above. The DNA was restricted with Spel, and the cleaved DNA was resolved by agarose gel electrophoresis. The restriction digestion with Spel cleaved the 3.6 kb amplification product into a 1.8 kb doublet band. This doublet band was cut from the gel and mixed with appropriately cut, gel purified pMALc or pET23b vector. These vectors were prepared by digestion with HindIII, filling in the overhanging ends using the Klenow enzyme, and cleaving with Xhal (pMALc) or Nhel (pET23b). The gel purified DNA fragments were purified using the Prep-A-Gene kit (BioRad) and the DNA was ligated, transformed and putative recombinant clones analyzed by restriction mapping.

pET and pMal clones containing the toxin B repeat insert (an interval 1750-2360 of toxin B) were verified by restriction mapping, using enzymes that cleaved specific sites within the toxin B region. In both cases fusion of the toxin B Spel site with either the compatible Abal site (pMal) or compatible Abal site (pET) is predicted to create an in frame fusion. This was confirmed in the case of the pMB1750-2360 clone by DNA sequencing of the clone

junction and 5' end of the toxin B insert using a MBP specific primer (New England Biolabs). In the case of the pET construct, the fusion of the blunt ended toxin B 3' end to the filled *Hind*III site should create an in-frame fusion with the C-terminal poly-histidine sequence in this vector. The pPB1750-2360 clone selected had lost, as predicted, the *Hind*III site at this clone junction; this eliminated the possibility that an additional adenosine residue was added to the 3' end of the PCR product by a terminal transferase activity of the *Pfit* polymerase, since fusion of this adenosine residue to the filled *Hind*III site would regenerate the restriction site (and was observed in several clones).

. 3

10

15

20

25

30

One liter cultures of each expression construct were grown, and fusion protein purified by affinity chromatography on either an amylose resin column (pMAL constructs; resin supplied by New England Biolabs) or Ni-chelate column (pET constructs; resin supplied by Qiagen or Novagen) as described [Williams et al. (1995), supra]. The integrity and purity of the fusion proteins were determined by Coomassie staining of SDS-PAGE protein gets as well as Western blot analysis with either an affinity purified goat polyclonal antiserum (Tech Lab), or a chicken polyclonal PEG prep. raised against the toxin B protein (CTB) as described above in Example 8. In both cases, affinity purification resulted in yields in excess of 20 mg protein per liter culture, of which greater than 90% was estimated to be full-length recombinant protein. It should be noted that the poly-histidine affinity tagged protein was released from the Qiagen Ni-NTA resin at low imidazole concentration (60 mM), necessitating the use of a 40 mM imidazole rather than a 60 mM imidazole wash step during purification.

A periplasmically secreted version of pMB1750-2360 was constructed by replacement of the promoter and MBP coding region of this construct with that from a related vector (pMAL ^{IM}-p2: New England Biolabs) in which a signal sequence is present at the N-terminus of the MBP, such that fusion protein is exported. This was accomplished by substituting a *BglII-EcoRV* promoter fragment from pMAL-p2 into pMB1750-2360. The yields of secreted, affinity purified protein (recovered from osmotic shock extracts as described by Riggs in *Current Protocols in Molecular Biology*, Vol. 2, Ausubel, *et al.*, Eds. (1989). Current Protocols, pp. 16.6.1 - 16.6.14] from this vector (pMBp1750-2360) were 6.5 mg/liter culture, of which 50% was estimated to be full-length fusion protein.

The interval was also expressed in two non-overlapping fragments. pMB1750-1970 was constructed by introduction of a frameshift into pMB1750-2360, by restriction with *Hind*III. filling in the overhanging ends and religation of the plasmid. Recombinant clones

were selected by loss of the *Hind*III site, and further restriction map analysis. Recombinant protein expression from this vector was more than 20 mg/liter of greater than 90% pure protein.

5

10

15

20

25

The complementary region was expressed in pMB1970-2360. This construct was created by removal of the 1750-1970 interval of pMB1750-2360. This was accomplished by restriction of this plasmid with *EcoRI* (in the pMalc polylinker 5° to the insert) and III. filling in the overhanging ends, and religation of the plasmid. The resultant plasmid, pMB1970-2360, was made using both intracellularly and secreted versions of the pMB1750-2360 vector.

No fusion protein was secreted in the pMBp1970-2360 version, perhaps due to a conformational constraint that prevents export of the fusion protein. However, the intracellularly expressed vector produced greater than 40mg/liter of greater than 90% full-length fusion protein.

Constructs to precisely express the toxin B repeats in either pMalc (pMB1850-2360) or pET16b (pPB1850-2360) were constructed as follows. The DNA interval including the toxin B repeats was PCR amplified as described above utilizing PCR primers P14 (SEQ ID NO:19) and P8 (SEQ ID NO:14). Primer P14 adds a *Eco*RI site immediately flanking the start of the toxin B repeats.

The amplified fragment was cloned into the pT7 Blue T-vector (Novagen) and recombinant clones in which single copies of the PCR fragment were inserted in either orientation were selected (pT71850-2360) and confirmed by restriction mapping. The insert was excised from two appropriately oriented independently isolated pT71850-2360 plasmids, with *Eco*R1 (5° end of repeats) and *Pst*1 (in the flanking polylinker of the vector), and cloned into *Eco*R1/*Pst*1 cleaved pMalc vector. The resulting construct (pMB1850-2360) was confirmed by restriction analysis, and yielded 20 mg/l of soluble fusion protein [greater than 90% intact (*i.e.*, nondegraded)] after affinity chromatography. Restriction of this plasmid with *Hind*111 and religation of the vector resulted in the removal of the 1970-2360 interval. The resultant construct (pMB1850-1970) expressed greater than 70 mg/liter of 90% full length fusion protein.

The pPB1850-2360 construct was made by cloning a *EcoRI* (filled with Klenow)-BamHI fragment from a pT71850-2360 vector (opposite orientation to that used in the pMB1850-2360 construction) into *NdeI* (filled)/BamHI cleaved pET16b vector. Yields of affinity purified soluble fusion protein were 15 mg/liter, of greater than 90% full length fusion protein.

. 5

10

15

20

25

30

Several smaller expression constructs from the 1750-2070 interval were also constructed in His-tagged pET vectors, but expression of these plasmids in the BL21 (DE3) host resulted in the production of high levels of mostly insoluble protein (see Table 23 and Figure 19). These constructs were made as follows.

pPB1850-1970 was constructed by cloning a *BglII-Hind*III fragment of pPB1850-2360 into *BglII/Hind*III cleaved pET23b vector. pPB1850-2070 was constructed by cloning a *BglII-PvaII* fragment of pPB1850-2360 into *BglII/Hinc*II cleaved pET23b vector. pPB1750-1970(c) was constructed by removal of the internal *Hind*III fragment of a pPB1750-2360 vector in which the vector *Hind*III site was regenerated during cloning (presumably by the addition of an A residue to the amplified PCR product by terminal transferase activity of *Pfu* polymerase). The pPB1750-1970(n) construct was made by insertion of the insert containing the *Ndel-Hind*III fragment of pPB1750-2360 into identically cleaved pETHisb vector. All constructs were confirmed by restriction digestion.

An expression construct that directs expression of the 10-470 aa interval of toxin B was constructed in the pMale vector as follows. A Nhel (a site 5' to the insert in the pET23 vector)-AflII (filled) fragment of the toxin B gene from pPB10-1530 was cloned into Xhal (compatible with Nhel)/HindIII (filled) pMale vector. The integrity of the construct (pMB10-470) was verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer (New England Biolabs). However, all expressed protein was degraded to the MBP monomer MW.

A second construct spanning this interval (aa 10-470) was constructed by cloning the PCR amplification product from a reaction containing the P9 (SEQ ID NO:15) and P10 (SEQ ID NO:16) primers (Figure 18) into the pETHisa vector. This was accomplished by cloning the PCR product as an *EcoR*1-blunt fragment into *EcoR*1-HincH restricted vector DNA: recombinant clones were verified by restriction mapping. Although this construct (pPB10-520) allowed expression and purification (utilizing the N-terminal polyhistidine affinity tag) of intact fusion protein, yields were estimated at less than 500 µg per liter culture.

Higher yield of recombinant protein from this interval (aa 10-520) were obtained by expression of the interval in two overlapping clones. The 10-330aa interval was cloned in both pETHisa and pMalc vectors, using the BamHI-AfIII (filled) DNA fragment from pPB10-520. This fragment was cloned into BamHI-HindII (filled) restricted pMalc or BamHI-HindII restricted pETHisa vector. Recombinant clones were verified by restriction mapping. High level expression of either insoluble (pET) or soluble (pMal) fusion protein was obtained. Total yields of fusion protein from the pMB10-330 construct (Figure 18) were 20 mg/liter culture, of which 10% was estimated to be full-length fusion protein. Although yields of this interval were higher and >90% full-length recombinant protein produced when expressed from the pET construct, the pMal fusion was utilized since the expressed protein was soluble and thus more likely to have the native conformation.

The pMB260-520 clone was constructed by cloning *Eco*R1-Xbal cleaved amplified DNA from a PCR reaction containing the P11 (SEQ ID NO:17) and P10 (SEQ ID NO:16) DNA primers (Figure 18) into similarly restricted pMalc vector. Yields of affinity purified protein were 10 mg/liter, of which approximately 50% was estimated to be full-length recombinant protein.

The aa510-1110 interval was expressed as described below. This entire interval was expressed as a pMal fusion by cloning the *Nhel-Hind*III fragment of pUCB10-1530 into *Xhal-Hind*III cleaved pMalc vector. The integrity of the construct (pMB510-1110) was verified by restriction mapping and DNA sequencing of the 5° clone junction using a MBP specific DNA primer. The yield of affinity purified protein was 25 mg/liter culture, of which 5% was estimated to be full-length fusion protein (1 mg/liter).

To attempt to obtain higher yields, this region was expressed in two fragments (aa510-820, and 820-1110) in the pMale vector. The pMB510-820 clone was constructed by insertion of a Sacl (in the pMale polylinker 5' to the insert)-Ilpal DNA fragment from pMB510-1110 into Sacl/Stal restricted pMale vector. The pMB820-1110 vector was constructed by insertion of the Hpal-IlindIII fragment of pUCB10-1530 into BamHI (filled)/HindIII cleaved pMale vector. The integrity of these constructs were verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer.

Recombinant protein expressed from the pMB510-820 vector was highly unstable. However, high levels (20 mg/liter) of >90% full-length fusion protein were obtained from the pMB820-1105 construct. The combination of partially degraded pMB510-1110 protein

30

5

10

15

20

25

(enriched for the 510-820 interval) with the pMB820-1110 protein provides usable amounts of recombinant antigen from this interval.

The aa1100-1750 interval was expressed as described below. The entire interval was expressed in the pMalc vector from a construct in which the Accl(filled)-Spel fragment of pPB10-1750 was inserted into Stul/Xbal (Xbal is compatible with Spel; Stul and filled Accl sites are both blunt ended) restricted pMalc. The integrity of this construct (pMB1100-1750) was verified by restriction mapping and DNA sequencing of the clone junction using a MBP specific DNA primer. Although 15 mg/liter of affinity purified protein was isolated from cells harboring this construct, the protein was greater than 99% degraded to MBP monomer size.

. 5

10

15

20

25

30

A smaller derivative of pMB1100-1750 was constructed by restriction of pMB1100-1750 with AflII and SalI (in the pMalc polylinker 3' to the insert), filling in the overhanging ends, and religating the plasmid. The resultant clone (verified by restriction digestion and DNA sequencing) has deleted the aa1530-1750 interval, was designated pMB1100-1530, pMB1100-1530 expressed recombinant protein at a yield of greater than 40 mg/liter, of which 5% was estimated to be full-length fusion protein.

Three constructs were made to express the remaining interval. Initially, a BspHI (filled)-Spel fragment from pPB10-1750 was cloned into EcoRI(filled)/Xbal cleaved pMalc vector. The integrity of this construct (pMB1570-1750) was verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer. Expression of recombinant protein from this plasmid was very low, approximately 3 mg affinity purified protein per liter, and most was degraded to MBP monomer size. This region was subsequently expressed from a PCR amplified DNA fragment. A PCR reaction utilizing primers P13 [SEQ ID NO:18: P13 was engineered to introduce an EcoRI site 5' to amplified toxin B sequences] and P8 (SEQ ID NO:14) was performed on C. difficile genomic DNA as described above. The amplified fragment was cleaved with EcoRI and Spel, and cloned into EcoRI/Abal cleaved pMalc vector. The resultant clone (pMB1530-1750) was verified by restriction map analysis, and recombinant protein was expressed and purified. The yield was greater than 20 mg protein per liter culture and it was estimated that 25% was full-length fusion protein; this was a significantly higher yield than the original pMB1570-1750 clone. The insert of pMB1530-1750 (in a EcoRI-Sall fragment) was transferred to the pETHisa vector (EcoRI/Xho1 cleaved, Xho1 and Sal1 ends are compatible). No detectable fusion protein was purified on Ni-Chelate columns from soluble lysates of cells induced to express fusion protein from this construct.

- 117 -

TABLE 23
Summary Of Toxin B Expression Constructs²

Clone	Affinity Tag	Yield (mg/liter)	% Full Length
pPB10-1750	none	low (estimated from Western blot hyb.)	?
pPB10-1530	none	low (as above)	ņ
pMB10-470	МВР	15mg/1	0%
pPB10-520	poly-his	0.5mg/l	20%
pPB10-330	poly-his	-20mg/l (insoluble)	90%
pMB10-330	MBP	20mg/l	10%
рМВ260-520	MBP	10mg/l	50%
pMB510-1110	MBP	25mg/l	5%
pMB510-820	МВР	degraded (by Western blot hyb)	
pMB820-1110	MBP	20mg/l	90%
pMB1100-1750	МВР	15mg/l	0%
pMB1100-1530	MBP	40mg/l	5%.
pMB1570-1750	МВР	3mg/l	- 5%
pPB1530-1750	poly-his	no purified protein detected	•
pMB1530-1~50	MBP	20mg/l	25%
pMB1=50-2360	MBP	·20mg/l	-90%
pMBp1750-2360	МВР	6.5mg/l (secreted)	50%
pPB1750-2360	poly-his	∵20mg/l	.9(1%
pMB1750-1970	МВР	·20mg/l	.90%
pMB1970-2360	МВР	40mg/l	-90%
pMBp1970-2360	МВР	(no secretion)	NA
pMB1850-2360	MBP	20mg/l	-90%
pPB1850-2360	poly-his	L5mg/l	-90%
pMB1850-1970	МВР	70mg/l	.90%
pPB1850-1970	poly-his	10mg/L (insoluble)	:-90%
pPB1850-2070	poly-his	·10mg/1 (insoluble)	90%
pPB1750-1970(c)	poly-his	10mg/l (insoluble)	-90%
pPB1750-1970(n)	poly-his	·10mg/l (insoluble)	90%

Clones in italics are clones currently utilized to purify recombinant protein from each selected interval.

30

5

10

15

20

25

occurs with the CTB antibody-recombinant mixture, that recombinant contains only weak or non-neutralizing epitopes of toxin B. This assay was performed as follows.

Antibodies against CTB were generated in egg laying Leghorn hens as described in Example 8. The lethal dosage (LD 100) of C. difficile toxin B when delivered I.P. into 40g female Golden Syrian hamsters (Charles River) was determined to be 2.5 to 5 µg. Antibodies generated against CTB and purified by PEG precipitation could completely protect the hamsters at the I.P. dosage determined above. The minimal amount of CTB antibody needed to afford good protection against 5 µg of CTB when injected I.P. into hamsters was also determined (1X PEG prep). These experiments defined the parameters needed to test whether a given recombinant protein could deplete protective CTB antibodies.

The cloned regions tested for neutralizing ability cover the entire toxin B gene and were designated as Intervals (INT) 1 through 5 (see Figure 19). Approximately equivalent final concentrations of each recombinant polypeptide were tested. The following recombinant polypeptides were used: 1) a mixture of intervals 1 and 2 (INT-1, 2); 2) a mixture of Intervals 4 and 5 (INT-4, 5) and 3) Interval 3 (INT-3). Recombinant proteins (each at about 1 mg total protein) were first preincubated with a final CTB antibody concentration of 1X [i.e., pellet dissolved in original yolk volume as described in Example 1(c)] in a final volume of 5 mls for 1 hour at 37°C. Twenty-five μg of CTB (at a concentration of 5 μg/ml), enough CTB to kill 5 hamsters, was then added and the mixture was then incubated for 1 hour at 37°C. Five, 40g female hamsters (Charles River) in each treatment group were then each given 1 ml of the mixture I.P. using a tuberculin syringe with a 27 gauge needle. The results of this experiment are shown in Table 24.

TABLE 24
Binding Of Neutralizing Antibodies By INT 3 Protein

Treatment Group	Number Of Animals Alive	Number Of Animals Dead
CTB antibodies	3)
CTB antibodies + 1NT1.2	3	2
CTB antibodies • INT4.5	3	3
CTB antibodies + INT 3	0	

C. difficile toxin B (CTB) was added to each group.

5

10

15

20

25

30

As shown in Table 24, the addition of recombinant proteins from INT-1, 2 or INT-4, 5 had no effect on the *in vivo* protective ability of the CTB antibody preparation compared to

EXAMPLE 19

Identification, Purification And Induction Of Neutralizing
Antibodies Against Recombinant C. difficile Toxin B Protein

To determine whether recombinant toxin B polypeptide fragments can generate neutralizing antibodies, typically animals would first be immunized with recombinant proteins and anti-recombinant antibodies are generated. These anti-recombinant protein antibodies are then tested for neutralizing ability in vivo or in vitro. Depending on the immunogenic nature of the recombinant polypeptide, the generation of high-titer antibodies against that protein may take several months. To accelerate this process and identify which recombinant polypeptide(s) may be the best candidate to generate neutralizing antibodies, depletion studies were performed. Specifically, recombinant toxin B polypeptide were pre-screened by testing whether they have the ability to bind to protective antibodies from a CTB antibody preparation and hence deplete those antibodies of their neutralizing capacity. Those recombinant polypeptides found to bind CTB, were then utilized to generate neutralizing antibodies. This Example involved: a) identification of recombinant sub-regions within toxin B to which neutralizing antibodies bind: b) identification of toxin B sub-region specific antibodies that neutralize toxin B in vivo; and c) generation and evaluation of antibodies reactive to recombinant toxin B polypeptides.

20

25

. 5

10

15

a) Identification Of Recombinant Sub-Regions Within Toxin B To Which Neutralizing Antibodies Bind

Sub-regions within toxin B to which neutralizing antibodies bind were identified by utilizing recombinant toxin B proteins to deplete protective antibodies from a polyclonal pool of antibodies against native C. difficile toxin B. An in vivo assay was developed to evaluate protein preparations for the ability to bind neutralizing antibodies. Recombinant proteins were first pre-mixed with antibodies directed against native toxin B (CTB antibody; see Example 8) and allowed to react for one hour at 37°C. Subsequently, C. difficile toxin B (CTB; Tech Lab) was added at a concentration lethal to hamsters and incubated for another hour at 37°C. After incubation this mixture was injected intraperitoneally (IP) into hamsters. If the recombinant polypeptide contains neutralizing epitopes, the CTB antibodies will lose its ability to protect the hamsters against death from CTB. If partial or complete protection

30

the CTB antibody preparation alone. In contrast, INT-3 recombinant polypeptide was able to remove all of the toxin B neutralizing ability of the CTB antibodies as demonstrated by the death of all the hamsters in that group.

The above experiment was repeated, using two smaller expressed fragments (pMB 1750-1970 and pMB 1970-2360, see Figure 19) comprising the INT-3 domain to determine if that domain could be further subdivided into smaller neutralizing epitopes. In addition, full-length INT-3 polypeptide expressed as a nickel tagged protein (pPB1750-2360) was tested for neutralizing ability and compared to the original INT-3 expressed MBP fusion (pMB1750-2360). The results are shown in Table 25.

TABLE 25

Removal Of Neutralizing Antibodies By Repeat Containing Proteins		
Treatment Group	Number Of Animals Alive	Number Of Animals Dead
CTB antibodies	.5	()
CTB antibodies + pPB1750-2360	0	5
CTB antibodies + pMB1750-2360	0	5
CTB antibodies + pMB1970-2360	3	2
CTB antibodies + pMB1750-1970	2	3

C. difficile toxin B (CTB) was added to each group.

20

. 5

10

15

25

30

The results summarized in Table 25 indicate that the smaller polypeptide fragments within the INT-3 domain, pMB1750-1970 and pMB1970-2360, partially lose the ability to bind to and remove neutralizing antibodies from the CTB antibody pool. These results demonstrate that the full length INT-3 polypeptide is required to completely deplete the CTB antibody pool of neutralizing antibodies. This experiment also shows that the neutralization epitope of INT-3 can be expressed in alternative vector systems and the results are independent of the vector utilized or the accompanying fusion partner.

Other Interval 3 specific proteins were subsequently tested for the ability to remove neutralizing antibodies within the CTB antibody pool as described above. The Interval 3 specific proteins used in these studies are summarized in Figure 23. In Figure 23 the following abbreviations are used: pP refers to the pET23 vector: pM refers to the pMALe vector: B refers to toxin B; the numbers refer to the amino acid interval expressed in the clone. The solid black ovals represent the MBP; and HHH represents the poly-histidine tag.

Only recombinant proteins comprising the entire toxin B repeat domain (pMB1750-2360, pPB1750-2360 and pPB1850-2360) can bind and completely remove neutralizing antibodies from the CTB antibody pool. Recombinant proteins comprising only a portion of the toxin B repeat domain were not capable of completely removing neutralizing antibodies from the CTB antibody pool (pMB1750-1970 and pMB1970-2360 could partially remove neutralizing antibodies while pMB1850-1970 and pPB1850-2070 failed to remove any neutralizing antibodies from the CTB antibody pool).

5

10

15

20

25

30

The above results demonstrate that only the complete ligand binding domain (repeat region) of the toxin B gene can bind and completely remove neutralizing antibodies from the CTB antibody pool. These results demonstrate that antibodies directed against the entire toxin B repeat region are necessary for *in vivo* toxin neutralization (see Figure 23: only the recombinant proteins expressed by the pMB1750-2360, pPB1750-2360 and pPB1850-2360 vectors are capable of completely removing the neutralizing antibodies from the CTB antibody pool).

These results represent the first indication that the entire repeat region of toxin B would be necessary for the generation of antibodies capable of neutralizing toxin B, and that sub-regions may not be sufficient to generate maximal titers of neutralizing antibodies.

b) Identification Of Toxin B Sub-Region Specific Antibodies That Neutralize Toxin B In Vivo

To determine if antibodies directed against the toxin B repeat region are <u>sufficient</u> for neutralization, region specific antibodies within the CTB antibody preparation were affinity purified, and tested for *in vivo* neutralization. Affinity columns containing recombinant toxin B repeat proteins were made as described below. A separate affinity column was prepared using each of the following recombinant toxin B repeat proteins: pPB1750-2360, pPB1850-2360, pMB1750-1970 and pMB1970-2360.

For each affinity column to be made, four ml of PBS-washed Actigel resin (Sterogene) was coupled overnight at room temperature with 5-10 mg of affinity purified recombinant protein (first extensively dialyzed into PBS) in 15 ml tubes (Falcon) containing a 1/10 final volume Ald-coupling solution (1 M sodium cyanoborohydride). Aliquots of the supernatants from the coupling reactions, before and after coupling, were assessed by Coomassic staining of 7.5% SDS-PAGE gels. Based on protein band intensities, in all cases greater than 30% coupling efficiencies were estimated. The resins were poured into 10 ml columns (BioRad),

washed extensively with PBS, pre-eluted with 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0) and reequilibrated in PBS. The columns were stored at 4°C.

- 5

10

15

20

25

Aliquots of a CTB IgY polyclonal antibody preparation (PEG prep) were affinity purified on each of the four columns as described below. The columns were hooked to a UV monitor (ISCO), washed with PBS and 40 ml aliquots of a 2X PEG prep (filter sterilized using a 0.45 µ filter) were applied. The columns were washed with PBS until the baseline value was re-established. The columns were then washed with BBStween to elute nonspecifically binding antibodies, and reequilibrated with PBS. Bound antibody was eluted from the column in 4M guanidine-HCl (in 10mM Tris-HCl, pH8.0). The cluted antibody was immediately dialyzed against a 100-fold excess of PBS at 4°C for 2 hrs. The samples were then dialyzed extensively against at least 2 changes of PBS, and affinity purified antibody was collected and stored at 4°C. The antibody preparations were quantified by UV absorbance. The clution volumes were in the range of 4-8 ml. All affinity purified stocks contained similar total antibody concentrations, ranging from 0.25-0.35% of the total protein applied to the columns.

The ability of the affinity purified antibody preparations to neutralize toxin B in vivo was determined using the assay outlined in a) above. Affinity purified antibody was diluted 1:1 in PBS before testing. The results are shown in Table 26.

In all cases similar levels of toxin neutralization was observed, such that lethality was delayed in all groups relative to preimmune controls. This result demonstrates that antibodies reactive to the repeat region of the toxin B gene are sufficient to neutralize toxin B in vivo. The hamsters will eventually die in all groups, but this death is maximally delayed with the CTB PEG prep antibodies. Thus neutralization with the affinity purified (AP) antibodies is not as complete as that observed with the CTB prep before affinity chromatography. This result may be due to loss of activity during guanidine denaturation (during the elution of the antibodies from the affinity column) or the presence of antibodies specific to other regions of the toxin B gene that can contribute to toxin neutralization (present in the CTB PEG prep).

WO 98/08540

TABLE 26

Neutralization Of Toxin B By Affinity Puritied Antibodies

Treatment group'	Number Animals Alive ^b	Number Animals Dead ^b
Preimmune ³	0	5
CTB': 400 μg	5	0
CTB (AP on pPB1750-2360): 875 µg	5	()
CTB (AP on pMB1750-1970):2 875 µg	5	0
CTB (AP on pMB1970-2360):2 500 µg	5	O

10

5

C' difficile toxin B (CTB) (Tech Lab: at 5 µg/ml, 25 µg total) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as either: '4X antibody PEG prep or 'affinity purified (AP) antibody (from CTB PEG prep, on indicated columns). The amount of specific antibody in each prep is indicated: the amount is directly determined for affinity purified preps and is estimated for the 4X CTB as described in Example 15.

15

The numbers in each group represent numbers of hamsters dead or alive, 2 hr post IP administration of toxin/antibody mixture.

20

25

The observation that antibodies affinity purified against the non-overlapping pMB1750-1970 and pMB1970-2360 proteins neutralized toxin B raised the possibility that either 1) antibodies specific to repeat sub-regions are sufficient to neutralize toxin B or 2) sub-region specific proteins can bind most or all repeat specific antibodies present in the CTB polyclonal pool. This would likely be due to conformational similarities between repeats, since homology in the primary amino acid sequences between different repeats is in the range of only 25-75% [Eichel-Streiber, et al. (1992) Molec. Gen. Genetics 233:260]. These possibilities were tested by affinity chromatography.

30

35

The CTB PEG prep was sequentially depleted 2X on the pMB1750-1970 column; only a small elution peak was observed after the second chromatography, indicating that most reactive antibodies were removed. This interval depleted CTB preparation was then chromatographed on the pPB1850-2360 column; no antibody bound to the column. The reactivity of the CTB and CTB (pMB1750-1970 depleted) preps to pPB1750-2360, pPB1850-2360, pMB1750-1970 and pMB1970-2360 proteins was then determined by ELISA using the protocol described in Example 13(c). Briefly, 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were coated with recombinant protein by adding 100 μl volumes of protein at 1-2 μg/ml in PBS containing 0.005% thimerosal to each well and incubating overnight at 4°C. The next morning, the coating suspensions were decanted and the wells were washed three

times using PBS. In order to block non-specific binding sites, 100 µl of 1.0% BSA (Sigma) in PBS (blocking solution) was then added to each well, and the plates were incubated for 1 hr. at 37°C. The blocking solution was decanted and duplicate samples of 150 µl of diluted antibody was added to the first well of a dilution series. The initial testing serum dilution was (1/200 for CTB prep. (the concentration of depleted CTB was standardized by OD₂₈₀) in blocking solution containing 0.5% Tween 20, followed by 5-fold serial dilutions into this solution. This was accomplished by serially transferring 30 µl aliquots to 120 µl buffer. mixing, and repeating the dilution into a fresh well. After the final dilution, 30 µl was removed from the well such that all wells contained 120 µl final volume. A total of 5 such dilutions were performed (4 wells total). The plates were incubated for 1 hr at 37°C. Following this incubation, the serially diluted samples were decanted and the wells were washed three times using PBS containing 0.5% Tween 20 (PBST), followed by two 5 min washes using BBS-Tween and a final three washes using PBST. To each well, 100 µl of 1 1000 diluted secondary antibody [rabbit anti-chicken IgG alkaline phosphatase (Sigma) diluted in blocking solution containing 0.5% Tween 20] was added, and the plate was incubated 1 hr at 37°C. The conjugate solutions were decanted and the plates were washed 6 times in PBST, then once in 50 mM Na₂CO₃, 10 mM MgCl₂, pH 9.5. The plates were developed by the addition of 100 µl of a solution containing 1 mg/ml para-nitro phenyl phosphate (Sigma) dissolved in 50 mM Na₂CO₃, 10 mM MgCl₂, pH9.5 to each well. The plates were then incubated at room temperature in the dark for 5-45 min. The absorbency of each well was measured at 410 nm using a Dynatech MR 700 plate reader.

• 5

10

15

20

25

30

As predicted by the affinity chromatography results, depletion of the CTB prep on the pMB1750-1970 column removed all detectable reactivity to the pMB1970-2360 protein. The reciprocal purification of a CTB prep that was depleted on the pMB1970-2360 column yielded no bound antibody when chromatographed on the pMB1750-1970 column. These results demonstrate that all repeat reactive antibodies in the CTB polyclonal pool recognize a conserved structure that is present in non-overlapping repeats. Although it is possible that this conserved structure represents rare conserved linear epitopes, it appears more likely that the neutralizing antibodies recognize a specific protein conformation. This conclusion was also suggested by the results of Western blot hybridization analysis of CTB reactivity to these recombinant proteins.

Western blots of 7.5% SDS-PAGE gels, loaded and electrophoresed with defined quantities of each recombinant protein, were probed with the CTB polyclonal antibody

preparation. The blots were prepared and developed with alkaline phosphatase as described in Example 3. The results are shown in Figure 24.

Figure 24 depicts a comparison of immunoreactivity of IgY antibody raised against either native or recombinant toxin B antigen. Equal amounts of pMB1750-1970 (lane 1), pMB1970-2360 (lane 2), pPB1850-2360 (lane 3) as well as a serial dilution of pPB1750-2360 (lanes 4-6 comprising 1X, 1/10X and 1/100X amounts, respectively) proteins were loaded in duplicate and resolved on a 7.5% SDS-PAGE gel. The gel was blotted and each half was hybridized with PEG prep IgY antibodies from chickens immunized with either native CTB or pPB1750-2360 protein. Note that the full-length pMB1750-1970 protein was identified only by antibodies reactive to the recombinant protein (arrows).

5

10

15

20

25

30

Although the CTB prep reacts with the pPB1750-2360, pPB1850-2360, and pMB1970-2360 proteins, no reactivity to the pMB1750-1970 protein was observed (Figure 24). Given that all repeat reactive antibodies can be bound by this protein during affinity chromatography, this result indicates that the protein cannot fold properly on Western blots. Since this eliminates all antibody reactivity, it is unlikely that the repeat reactive antibodies in the CTB prep recognize linear epitopes. This may indicate that in order to induce protective antibodies, recombinant toxin B protein will need to be properly folded.

c) Generation And Evaluation Of Antibodies Reactive To Recombinant Toxin B Polypeptides

i) Generation Of Antibodies Reactive To Recombinant Toxin B Proteins

Antibodies against recombinant proteins were generated in egg laying Leghorn hens as described in Example 13. Antibodies were raised (using Freunds adjuvant (Gibco) unless otherwise indicated] against the following recombinant proteins: 1) a mixture of Interval 1+2 proteins (see Figure 18); 2) a mixture of interval 4 and 5 proteins (see Figure 18); 3) pMB1970-2360 protein; 4) pPB1750-2360 protein; 5) pMB1750-2360; 6) pMB1750-2360 [Titermax adjuvant (Vaxcell)]; 7) pMB1750-2360 [Gerbu adjuvant (Biotech)]; 8) pMBp1750-2360 protein; 9) pPB1850-2360; and 10) pMB1850-2360.

Chickens were boosted at least 3 times with recombinant protein until ELISA reactivity [using the protocol described in b) above with the exception that the plates were coated with pPB1750-2360 protein] of polyclonal PEG preps was at least equal to that of the CTB polyclonal antibody PEG prep. ELISA titers were determined for the PEG preps from

all of the above immunogens and were found to be comparable ranging from 1:12500 to 1:62500. High titers were achieved in all cases except in 6) pMB1750-2360 in which strong titers were not observed using the Titermax adjuvant, and this preparation was not tested further.

• 5

ii) Evaluation Of Antibodies Reactive To Recombinant Proteins By Western Blot Hybridization

10

Western blots of 7.5% SDS-PAGE gels, loaded and electrophoresed with defined quantities of recombinant protein (pMB1750-1970, pPB1850-2360, and pMB1970-2360 proteins and a serial dilution of the pPB1750-2360 to allow quantification of reactivity), were probed with the CTB, pPB1750-2360, pMB1750-2360 and pMB1970-2360 polyclonal antibody preparations (from chickens immunized using Freunds adjuvant). The blots were prepared and developed with alkaline phosphatase as described above in b).

15

As shown in Figure 24, the CTB and pMB1970-2360 preps reacted strongly with the pPB1750-2360, pPB1850-2360, and pMB1970-2360 proteins while the pPB1750-2360 and pMB1970-2360 (Gerbu) preparations reacted strongly with all four proteins. The Western blot reactivity of the pPB1750-2360 and pMB1970-2360 (Gerbu) preparations were equivalent to that of the CTB preparation, while reactivity of the pMB1970-2360 preparation was <10% that of the CTB prep. Despite equivalent ELISA reactivities only weak reactivity (approximately 1%) to the recombinant proteins were observed in PEG preps from two independent groups immunized with the pMB1750-2360 protein and one group immunized with the pMB1750-2360 preparation using Freunds adiuvant.

25

30

20

Affinity purification was utilized to determine if this difference in immunoreactivity by Western blot analysis reflects differing antibody titers. Fifty ml 2X PEG preparations from chickens immunized with either pMB1750-2360 or pMB1970-2360 protein were chromatographed on the pPB1750-2360 affinity column from b) above, as described. The yield of affinity purified antibody (% total protein in preparation) was equivalent to the yield obtained from a CTB PEG preparation in b) above. Thus, differences in Western reactivity reflect a qualitative difference in the antibody pools, rather than quantitative differences.. These results demonstrate that certain recombinant proteins are more effective at generating high affinity antibodies (as assayed by Western blot hybridization).

iii) In Vivo Neutralization Of Toxin B Using Antib dies Reactive To Recombinant Protein

5

10

15

20

The *in vivo* hamster model [described in Examples 9 and 14(b)] was utilized to assess the neutralizing ability of antibodies raised against recombinant toxin B proteins. The results from three experiments are shown below in Tables 27-29.

The ability of each immunogen to neutralize toxin B in vivo has been compiled and is shown in Table 30. As predicted from the recombinant protein-CTB premix studies (Table 24) only antibodies to Interval 3 (1750-2366) and not the other regions of toxin B (i.e., intervals 1-5) are protective. Unexpectedly, antibodies generated to INT-3 region expressed in pMAL vector (pMB1750-2360 and pMpB1750-2360) using Freunds adjuvant were nonneutralizing. This observation is reproducible, since no neutralization was observed in two independent immunizations with pMB1750-2360 and one immunization with pMpB1750-2360. The fact that 5X quantities of affinity purified toxin B repeat specific antibodies from pMB1750-2360 PEG preps cannot neutralize toxin B while 1X quantities of affinity purified anti-CTB antibodies can (Table 28) demonstrates that the differential ability of CTB antibodies to neutralize toxin B is due to qualitative rather than quantitative differences in these antibody preparations. Only when this region was expressed in an alternative vector (pPB1750-2360) or using an alternative adjuvant with the pMB1750-2360 protein were neutralizing antibodies generated. Importantly, antibodies raised using Freunds adjuvant to pPB1850-2360, which contains a fragment that is only 100 amino acids smaller than recombinant pPB1750-2360, are unable to neutralize toxin B in vivo (Table 27); note also that the same vector is used for both pPB1850-2360 and pPB1750-2360.

. 5

10

15

20

25

30

35

TABLE 27
In Vivo Neutralization Of Toxin B

Treatment Group	Number Animals Alive ^h	Number Animals Dead
Preimmune	0	
СТВ	5	0
INT1+2	0	5
INT 4+5	0	<u></u>
pMB1750-2360	0	
pMB1970-2360	0	
pPB1750-2360	5	

C. difficile toxin B (CTB) (at 5 µg/ml; 25 µg total; Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as a 4X antibody PEG prep.

The numbers in each group represent numbers of hamsters dead or alive, 2 hours post IP administration of toxin/antibody mixture.

TABLE 28

Treatment Group*	Number Animals Alive ^h	Number Animals Dead
Preimmune(1)	0	5
CTB(1)	5	0
pPB1750-2360(1)	5	0
L5 mg anti-pMB1750-2360(2)		1
1.5 mg anti-pMB1970-2360(2)	0	
300 μg anti-CTB(2)	5	^

C. difficile toxin B (CTB) (at 5 µg/ml; 25 µg total; Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation, 1 ml of this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as either (1) 4X antibody PEG prep or (2) affinity purified antibody (on a pPB1750-2360 resin), either 1.5 mg/group (anti-pMB1750-2360 and anti-pMB1970-2360; used undiluted affinity purified antibody) or 350 µg/group (anti-CTB, repeat specific; used 1/5 diluted anti-CTB antibody).

The numbers in each group represent numbers of hamsters dead or alive, 2 hr post-IP administration of toxin/antibody mixture.

TABLE 29

Generation Of Neutralizing Antibodies Utilizing The Gerbu Adjuvant

Treatment Group	Number Animals Alive ^b	Number Animals Dead
Preimmune	0	5
СТВ	5	0
pMB1970-2360	0	5
pMB1850-2360	0	5
pPB1850-2360	0	5
pMB1750-2360 (Gerbu adj)	5	0

5

10

15

C. difficile toxin B (CTB) (Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as a 4X antibody PEG prep.

The numbers in each group represent numbers of hamsters dead or alive. 2hrs post IP administration of toxin antibody mixture.

TABLE 30
In Vivo Neutralization Of Toxin B

lınmunogen	Adjuvanı	Tested Preparation*	Antigen Utilized For AP	<i>In vivo</i> Neutralization
Preimmune	NA,	PEG	NA	no
CTB (native)	Titermax	PEG	NA	ves
CTB (native)	Titermax	AP	pPB1750-2360	
CTB (native)	Titermax	AP	pPB1850-2360	yes
CTB (native)	Titermax	AP	pPB1750-1970	yes
CTB (native)	Titermax	АР	pPB1970-2360	yes
pMB1750-2360	Freunds	PEG	NA	no
pMB1750-2360	Freunds	AP	pPB1750-2360	no
pMB1750-2360	Gerbu	PEG	NA	yes
pMB1970-2360	Freunds	PEG	NA	no
pMB1970-2360	Freunds	AP	pPB1750-2360	no
pPB1750-2360	Freunds	PEG	NA	yes
pPB1850-2360	Freunds	PEG	NA	no
pMB1850-2360	Freunds	PEG	NA	no
INT 1-2	Freunds	PEG	NA	no
INT 4+5	Freunds	PEG	NA	no

Either PFG preparation (PEG) or affinity purified antibodies (AP).

'Yes' denotes complete neutralization (0/5 dead) while 'no' denotes no neutralization (5/5 dead) of toxin B. 2 hours post-administration of mixture.

'NA' denotes not applicable.

10

15

20

25

30

35

The pPB1750-2360 antibody pool confers significant *in vivo* protection, equivalent to that obtained with the affinity purified CTB antibodies. This correlates with the observed high affinity of this antibody pool (relative to the pMB1750-2360 or pMB1970-2360 pools) as assayed by Western blot analysis (Figure 24). These results provide the first demonstration that *in vivo* neutralizing antibodies can be induced using recombinant toxin B protein as immunogen.

The failure of high concentrations of antibodies raised against the pMB1750-2360 protein (using Freunds adjuvant) to neutralize, while the use of Gerbu adjuvant and pMB1750-2360 protein generates a neutralizing response, demonstrates that conformation or presentation of this protein is essential for the induction of neutralizing antibodies. These

results are consistent with the observation that the neutralizing antibodies produced when native CTB is used as an immunogen appear to recognize conformational epitopes [see section b) above]. This is the first demonstration that the conformation or presentation of recombinant toxin B protein is essential to generate high titers of neutralizing antibodies.

5

EXAMPLE 20

Determination Of Quantitative And Qualitative
Differences Between pMB1750-2360, pMB1750-2360 (Gerbu)
Or pPB1750-2360 lgY Polyclonal Antibody Preparations

10

15

In Example 19, it was demonstrated that toxin B neutralizing antibodies could be generated using specific recombinant toxin B proteins (pPB1750-2360) or specific adjuvants. Antibodies raised against pMB1750-2360 were capable of neutralizing the enterotoxin effect of toxin B when the recombinant protein was used to immunize hens in conjunction with the Gerbu adjuvant, but not when Freunds adjuvant was used. To determine the basis for these antigen and adjuvant restrictions, toxin B-specific antibodies present in the neutralizing and non-neutralizing PEG preparations were isolated by affinity chromatography and tested for qualitative or quantitative differences. The example involved a) purification of anti-toxin B specific antibodies from pMB1750-2360 and pPB1750-2360 PEG preparations and b) in vivo neutralization of toxin B using the affinity purified antibody.

20

a) Purification Of specific Antibodies From pMB1750-2360 And pPB1750-2360 PEG Preparations

25

To purify and determine the concentration of specific antibodies (expressed as the percent of total antibody) within the pPB1750-2360 (Freunds and Gerbu) and pPB1750-2360 PEG preparations, defined quantities of these antibody preparations were chromatographed on an affinity column containing the entire toxin B repeat region (pPB1750-2360). The amount of affinity purified antibody was then quantified.

30

An affinity column containing the recombinant toxin B repeat protein, pPB1750-2360, was made as follows. Four ml of PBS-washed Actigel resin (Sterogene) was coupled with 5 mg of pPB1750-2360 affinity purified protein (dialyzed into PBS: estimated to be greater than 95% full length fusion protein) in a 15 ml tube (Falcon) containing 1/10 final volume Ald-coupling solution (1M sodium cyanoborohydride). Aliquots of the supernatant from the

coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, greater than 95% (approximately 5 mg) of recombinant protein was coupled to the resin. The coupled resin was poured into a 10 ml column (BioRad), washed extensively with PBS, pre-cluted with 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0: 0.005% thimerosal) and re-equilibrated in PBS and stored at 4°C.

. 5 .

10

15

20

25

30

Aliquots of pMB1750-2360, pMB1750-2360 (Gerbu) or pPB1750-2360 IgY polyclonal antibody preparations (PEG preps) were affinity purified on the above column as follows. The column was attached to an UV monitor (ISCO), and washed with PBS. Forty ml aliquots of 2X PEG preps (filter sterilized using a 0.45 μ filter and quantified by OD₂₈₀ before chromatography) was applied. The column was washed with PBS until the baseline was reestablished (the column flow-through was saved), washed with BBSTween to clute nonspecifically binding antibodies and re-equilibrated with PBS. Bound antibody was cluted from the column in 4M guanidine-HCl (in 10 mM Tris-HCL, pH 8.0, 0.005% thimerosal) and the entire elution peak collected in a 15 ml tube (Falcon). The column was re-equilibrated, and the column cluate re-chromatographed as described above. The antibody preparations were quantified by UV absorbance (the elution buffer was used to zero the spectrophotometer). Approximately 10 fold higher concentrations of total purified antibody was obtained upon clution of the first chromatography pass relative to the second pass. The low yield from the second chromatography pass indicated that most of the specific antibodies were removed by the first round of chromatography.

Pools of affinity purified specific antibodies were prepared by dialysis of the column elutes after the first column chromatography pass for the pMB1750-2360, pMB1750-2360 (Gerbu) or pPB1750-2360 IgY polyclonal antibody preparations. The elutes were collected on ice and immediately dialyzed against a 100-fold volume of PBS at 4°C for 2 hrs. The samples were then dialyzed against 3 changes of a 65-fold volume of PBS at 4°C. Dialysis was performed for a minimum of 8 hrs per change of PBS. The dialyzed samples were collected, centrifuged to remove insoluble debris, quantified by OD₂₈₀, and stored at 4°C.

The percentage of toxin B repeat-specific antibodies present in each preparation was determined using the quantifications of antibody yields from the first column pass (amount of specific antibody recovered after first pass/total protein loaded). The yield of repeat-specific affinity purified antibody (expressed as the percent of total protein in the preparation) in: 1) the pMB1750-2360 PEG prep was approximately 0.5%, 2) the pMB1750-2360 (Gerbu) prep was approximately 2.3%, and 3) the pPB1750-2360 prep was approximately 0.4%,

Purification of a CTB IgY polyclonal antibody preparation on the same column demonstrated that the concentration of toxin B repeat specific antibodies in the CTB preparation was 0.35%.

These results demonstrate that 1) the use of Gerbu adjuvant enhanced the titer of specific antibody produced against the pMB1750-2360 protein 5-fold relative to immunization using Freunds adjuvant, and 2) the differences seen in the *in vivo* neutralization ability of the pMB1750-2360 (not neutralizing) and pPB1750-2360 (neutralizing) and CTB (neutralizing) PEG preps seen in Example 19 was not due to differences in the titers of repeat-specific antibodies in the three preparations because the titer of repeat-specific antibody was similar for all three preps; therefore the differing ability of the three antibody preparations to neutralize toxin B must reflect qualitative differences in the induced toxin B repeat-specific antibodies. To confirm that qualitative differences exist between antibodies raised in hens immunized with different recombinant proteins and/or different adjuvants, the same amount of affinity purified anti-toxin B repeat (aa 1870-2360 of toxin B) antibodies from the different preparations was administered to hamsters using the *in vivo* hamster model as described below.

b) In vivo Neutralization Of Toxin B Using Affinity Purified Antibody

The *in vivo* hamster model was utilized to assess the neutralizing ability of the affinity purified antibodies raised against recombinant toxin B proteins purified in (a) above. As well, a 4X IgY PEG preparation from a second independent immunization utilizing the pPB1750-2360 antigen with Freunds adjuvant was tested for *in vivo* neutralization. The results are shown in Table 31.

The results shown in Table 31 demonstrate that:

5

10

15

20

25

30

as shown in Example 19 and reproduced here. 1.5 mg of affinity purified antibody from pMB1750-2360 immunized hens using Freunds adjuvant does not neutralize toxin B *in vivo*. However, 300 µg of affinity purified antibody from similarly immunized hens utilizing Gerbu adjuvant demonstrated complete neutralization of toxin B *in vivo*. This demonstrates that Gerbu adjuvant, in addition to enhancing the titer of antibodies reactive to the pMB1750-2360 antigen relative to Freunds adjuvant (demonstrated in (a) above), also enhances the yield of neutralizing antibodies to this antigen, greater than 5 fold.

Complete *in vivo* neutralization of toxin B was observed with 1.5 mg of affinity purified antibody from hens immunized with pPB1750-2360 antigen. but not with pMB1750-2360 antigen, when Freunds adjuvant was used. This demonstrates, using standardized toxin B repeat-specific antibody concentrations, that neutralizing antibodies were induced when pPB1750-2360 but not pMB1750-2360 was used as the antigen with Freunds adjuvant.

5

10

15

- Complete *in vivo* neutralization was observed with 300 μg of pMB1750-2360 (Gerbu) antibody, but not with 300 μg of pPB1750-2360 (Freunds) antibody. Thus the pMB1750-2360 (Gerbu) antibody has a higher titer of neutralizing antibodies than the pPB1750-2360 (Freunds) antibody.
- 4) Complete neutralization of toxin B was observed using 300 µg of CTB antibody [affinity purified (AP)] but not 100 µg CTB antibody (AP or PEG prep). This demonstrates that greater than 100 µg of toxin B repeat-specific antibody (anti-CTB) is necessary to neutralize 25 µg toxin B *in vivo* in this assay, and that affinity purified antibodies specific to the toxin B repeat interval neutralize toxin B as effectively as the PEP prep of IgY raised against the entire CTB protein (shown in this assay).
- As was observed with the initial pPB1750-2360 (IgY) PEG preparation (Example 19), complete neutralization was observed with a IgY PEG preparation isolated from a second independent group of pPB1750-2360 (Freunds) immunized hens. This demonstrates that neutralizing antibodies are reproducibly produced when hens are immunized with pPB1750-2360 protein utilizing Freunds adjuvant.

TABLE 31

In vivo Neutralization Of Toxin B Using Affinity Purified Antibodies

Treatment Groups	Number Animals Alive	Number Animals Deadb
Preimmune ¹	0	5
CTB (300 μg) ²	5	0
CTB (100 μg) ²	ı	4
pMB1750-2360 (G) (5 mg) ²	5	0
pMB1750-2360 (G) (1.5 mg) ²	5	o o
pMB1750-2360 (G) (300 μg) ²	5	0
pMB1750-2360 (F) (1.5 mg) ²	0	5
pPB1750-2360 (F) (1.5 mg) ²	5	0
pPB1750-2360 (F) (300 μg) ²	I	+
CTB (100 μg)	2	3
pPB1750-2360 (F) (500 μg)	5	()

5

10

15

20

25

30

35

C. difficile toxin B (CTB) (Tech Lab) at lethal concentration to hamsters (25 µg) was added to the antibody (amount of specific antibody is indicated) and incubated for one hour at 37°C. After incubation, this mixture was injected IP into hamsters (1/5 total mix injected per hamster). Each treatment group received toxin premixed with antibody raised against the indicated protein (Gegerbu adjuvant, F=Freunds adjuvant). Indicates the antibody was a 4X IgY PEG prep: Indicates the antibody was affinity purified on a pPB1850-2360 result and indicates that the antibody was a 1X IgY PEG prep.

The numbers in each group represent numbers of hamsters dead or alive, 2 hrs post IP administration of toxin/antibody mixture.

EXAMPLE 21

Diagnostic Enzyme Immunoassays For C. difficile Toxins A And B

The ability of the recombinant toxin proteins and antibodies raised against these recombinant proteins (described in the above examples) to form the basis of diagnostic assays for the detection of clostridial toxin in a sample was examined. Two immunoassay formats were tested to quantitatively detect *C. difficile* toxin A and toxin B from a biological specimen. The first format involved a competitive assay in which a fixed amount of recombinant toxin A or B was immobilized on a solid support (e.g., microtiter plate wells) followed by the addition of a toxin-containing biological specimen mixed with affinity-purified or PEG fractionated antibodies against recombinant toxin A or B. If toxin is present in a specimen, this toxin will compete with the immobilized recombinant toxin protein for

binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of a reporter reagent. The reporter reagent detects the presence of antibody bound to the immobilized toxin protein.

In the second format, a sandwich immunoassay was developed using affinity-purified antibodies to recombinant toxin A and B. The affinity-purified antibodies to recombinant toxin A and B were used to coat microtiter wells instead of the recombinant polypeptides (as was done in the competitive assay format). Biological samples containing toxin A or B were then added to the wells followed by the addition of a reporter reagent to detect the presence of bound toxin in the well.

10

15

20

25

30

- 5

a) Competitive Immunoassay For The Detection Of C. difficile Toxin

Recombinant toxin A or B was attached to a solid support by coating 96 well microtiter plates with the toxin protein at a concentration of Lµg/ml in PBS. The plates were incubated overnight at 2-8°C. The following morning, the coating solutions were removed and the remaining protein binding sites on the wells were blocked by filling each well with a PBS solution containing 0.5% BSA and 0.05% Tween-20. Native C. difficile toxin A or B (Tech Lab) was diluted to 4 μg/ml in stool extracts from healthy Syrian hamsters (Sasco). The stool extracts were made by placing fecal pellets in a 15 ml centrifuge tube: PBS was added at 2 ml/pellet and the tube was vortexed to create a uniform suspension. The tube was then centrifuged at 2000 rpm for 5 min at room temperature. The supernatant was removed: this comprises the stool extract. Fifty µl of the hamster stool extract was pipetted into each well of the microtiter plates to serve as the diluent for serial dilutions of the 4 $\mu g/ml$ toxin samples. One hundred μl of the toxin samples at 4 $\mu g/ml$ was pipetted into the first row of wells in the microtiter plate, and $50~\mu l$ aliquots were removed and diluted serially down the plate in duplicate. An equal volume of affinity purified anti-recombinant toxin antibodies [1 ng/well of anti-pMA1870-2680 antibody was used for the detection of toxin A: 0.5 ng/well of anti-pMB1750-2360(Gerbu) was used for the detection of toxin B] were added to appropriate wells, and the plates were incubated at room temperature for 2 hours with gentle agitation. Wells serving as negative control contained antibody but no native toxin to compete for binding.

Unbound toxin and antibody were removed by washing the plates 3 to 5 times with PBS containing 0.05% Tween-20. Following the wash step, 100 μ l of rabbit anti-chicken IgG

antibody conjugated to alkaline phosphatase (Sigma) was added to each well and the plates were incubated for 2 hours at room temperature. The plates were then washed as before to remove unbound secondary antibody. Freshly prepared alkaline phosphatase substrate (1 mg/ml p-nitrophenyl phosphate (Sigma) in 50 mM Na₂CO₃, pH 9.5: 10 mM MgCl₂) was added to each well. Once sufficient color developed, the plates were read on a Dynatech MR700 microtiter plate reader using a 410 nm filter.

5

10

15

20

25

The results are summarized in Tables 32 and 33. For the results shown in Table 32, the wells were coated with recombinant toxin A protein (pMA1870-2680). The amount of native toxin A added (present as an addition to solubilized hamster stool) to a given well is indicated (0 to 200 ng). Antibody raised against the recombinant toxin A protein, pMA1870-2680, was affinity purified on the an affinity column containing pPA1870-2680 (described in Example 20). As shown in Table 32, the recombinant toxin A protein and affinity-purified antitoxin can be used for the basis of a competitive immunoassay for the detection of toxin A in biological samples.

Similar results were obtained using the recombinant toxin B. pPB1750-2360, and antibodies raised against pMB1750-2360(Gerbu). For the results shown in Table 33, the wells were coated with recombinant toxin B protein (pPB1750-2360). The amount of native toxin B added (present as an addition to solubilized hamster stool) to a given well is indicated (0 to 200 ng). Antibody raised against the recombinant toxin B protein, pMB1750-2360(Gerbu), was affinity purified on the an affinity column containing pPB1850-2360 (described in Example 20). As shown in Table 33, the recombinant toxin B protein and affinity-purified antitoxin can be used for the basis of a competitive immunoassay for the

In this competition assay, the reduction is considered significant over the background levels at all points; therefore the assay can be used to detect samples containing less than 12.5 ng toxin A/well and as little as 50-100 ng toxin B/well.

detection of toxin B in biological samples.

TABLE 32

Competitive Inhibition Of Anti-C. difficile Toxin A By Native Toxin A

ng Toxin A/Well	OD Product
	OD ₁₁₀ Readout
200	0.176
100	0.253
50	0.240
25	0.259
12.5	0.309
6.25	0.367
3.125	0.417
0	0.590

• 5

10

15

20

25

30

TABLE 33

Competitive Inhibition Of Anti-C. difficule Toxin B By Native Toxin B

ng Toxin B/Well	OD ₁₁₀ Readout
200	0.392
100	0.566
50	0.607
25	0.778
12.5	0 970
6.25	0.902
3.125	1.040
0	1.055

These competitive inhibition assays demonstrate that native *C. difficile* toxins and recombinant *C. difficile* toxin proteins can compete for binding to antibodies raised against recombinant *C. difficile* toxins demonstrating that these anti-recombinant toxin antibodies provide effective diagnostic reagents.

b) Sandwich Immunoassay For The Detection Of C. difficile Toxin

Affinity-purified antibodies against recombinant toxin A or toxin B were immobilized to 96 well microtiter plates as follows. The wells were passively coated overnight at 4°C with affinity purified antibodies raised against either pMA1870-2680 (toxin A) or pMB1750-

2360(Gerbu) (toxin B). The antibodies were affinity purified as described in Example 20. The antibodies were used at a concentration of 1 µg/ml and 100 µl was added to each microtiter well. The wells were then blocked with 200 µl of 0.5% BSA in PBS for 2 hours at room temperature and the blocking solution was then decanted. Stool samples from healthy Syrian hamsters were resuspended in PBS, pH 7.4 (2 ml PBS/stool pellet was used to resuspend the pellets and the sample was centrifuged as described above). The stool suspension was then spiked with native C. difficile toxin A or B (Tech Lab) at 4 µg/ml. The stool suspensions containing toxin (either toxin A or toxin B) were then serially diluted two-fold in stool suspension without toxin and 50 µl was added in duplicate to the coated microtiter wells. Wells containing stool suspension without toxin served as the negative control.

The plates were incubated for 2 hours at room temperature and then were washed three times with PBS. One hundred µl of either goat anti-native toxin A or goat anti-native toxin B (Tech Lab) diluted 1:1000 in PBS containing 1% BSA and 0.05% Tween 20 was added to each well. The plates were incubated for another 2 hours at room temperature. The plates were then washed as before and 100 µl of alkaline phosphatase-conjugated rabbit anti-goat lgG (Cappel, Durham, N.C.) was added at a dilution of 1:1000. The plates were incubated for another 2 hours at room temperature. The plates were washed as before then developed by the addition of 100 µl/well of a substrate solution containing 1 mg/ml p-nitrophenyl phosphate (Sigma) in 50 mM Na₂CO₃, pH 9.5; 10 mM MgCl₃. The absorbance of each well was measured using a plate reader (Dynatech) at 410 nm. The assay results are shown in Tables 34 and 35.

TABLE 34

C. difficile Toxin A Detection In Stool Using Affinity-Purified Antibodies Against Toxin A

	Total Research	
ng Toxin A/Well	OD ₄₁₀ Readout	
200	0.9	
100	0.8	
50	0.73	
25	0.71	
12.5	0.59	
6.25	0.421	
0	()	

30

25

5

10

15

20

TABLE 35

C. difficile Toxin B Detection In Stool Using Affinity-Purified Antibodies Against Toxin B

ng Toxin B/Well	OD ₁₁₀ Readout
200	1.2
100	0.973
50	0.887
25	0.846
12.5	0.651
6.25	0.431
0	0.004

5

10

15

20

25

30

The results shown in Tables 34 and 35 show that antibodies raised against recombinant toxin A and toxin B fragments can be used to detect the presence of C. difficile toxin in stool samples. These antibodies form the basis for a sensitive sandwich immunoassay which is capable of detecting as little as 6.25 ng of either toxin A or B in a 50 µl stool sample. As shown above in Tables 34 and 35, the background for this sandwich immunoassay is extremely low: therefore, the sensitivity of this assay is much lower than 6.25 ng toxin/well. It is likely that toxin levels of 0.5 to 1.0 pg/well could be detected by this assay.

The results shown above in Tables 32-35 demonstrate clear utility of the recombinant reagents in C. difficile toxin detection systems.

EXAMPLE 22

Construction And Expression Of C. botulinum C Fragment Fusion Proteins

The C. hotulinum type A neurotoxin gene has been cloned and sequenced [Thompson, et al., Eur. J. Biochem, 189:73 (1990)]. The nucleotide sequence of the toxin gene is available from the EMBL/GenBank sequence data banks under the accession number X52066: the nucleotide sequence of the coding region is listed in SEQ ID NO:27. The amino acid sequence of the C. hotulinum type A neurotoxin is listed in SEQ ID NO:28. The type A neurotoxin gene is synthesized as a single polypeptide chain which is processed to form a dimer composed of a light and a heavy chain linked via disulfide bonds. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H_C domain.

Previous attempts by others to express polypeptides comprising the C fragment of C. botulinum type A toxin as a native polypeptide (e.g., not as a fusion protein) in E. coli have

been unsuccessful [H.F. LaPenotiere. et al. in Botulinum and Tetanus Neurotoxins. DasGupta. Ed., Plenum Press, New York (1993), pp. 463-466]. Expression of the C fragment as a fusion with the E. coli MBP was reported to result in the production of insoluble protein (H.F. LaPenotiere, et al., supra).

5

In order to produce soluble recombinant C fragment proteins in E. coli, fusion proteins comprising a synthetic C fragment gene derived from the C. botulinum type A toxin and either a portion of the C. difficile toxin protein or the MBP were constructed. This example involved a) the construction of plasmids encoding C fragment fusion proteins and b) expression of C. botulinum C fragment fusion proteins in E. coli.

10

15

a) Construction Of Plasmids Encoding C Fragment Fusion Proteins

In Example 11, it was demonstrated that the *C. difficile* toxin A repeat domain can be efficiently expressed and purified in *E. coli* as either native (expressed in the pET 23a vector in clone pPA1870-2680) or fusion (expressed in the pMALc vector as a fusion with the *E. coli* MBP in clone pMA1870-2680) proteins. Fusion proteins comprising a fusion between the MBP, portions of the *C. difficile* toxin A repeat domain (shown to be expressed as a soluble fusion protein) and the C fragment of the *C. botulinum* type A toxin were constructed. A fusion protein comprising the C fragment of the *C. botulinum* type A toxin and the MBP was also constructed.

20

Figure 25 provides a schematic representation of the botulinal fusion proteins along with the donor constructs containing the *C. difficile* toxin A sequences or *C. botulinum C* fragment sequences which were used to generate the botulinal fusion proteins. In Figure 25, the solid boxes represent *C. difficile* toxin A gene sequences, the open boxes represent *C. botulinum C* fragment sequences and the solid black ovals represent the *E. coli* MBP. When the name for a restriction enzyme appears inside parenthesis, this indicates that the restriction site was destroyed during construction. An asterisk appearing with the name for a restriction enzyme indicates that this restriction site was recreated at the cloning junction.

30

25

In Figure 25, a restriction map of the pMA1870-2680 and pPA1100-2680 constructs (described in Example 11) which contain sequences derived from the *C. difficile* toxin A repeat domain are shown; these constructs were used as the source of *C. difficile* toxin A gene sequences for the construction of plasmids encoding fusions between the *C. botulinum C* fragment gene and the *C. difficile* toxin A gene. The pMA1870-2680 expression construct

expresses high levels of soluble, intact fusion protein (20 mg/liter culture) which can be affinity purified on an amylose column (purification described in Example 11d).

. 5

10

15

20

25

30

The pAlterBot construct (Figure 25) was used as the source of *C. horulinum* C fragment gene sequences for the botulinal fusion proteins. pAlterBot was obtained from J. Middlebrook and R. Lemley at the U.S. Department of Defense. pAlterBot contains a synthetic *C. horulinum* C fragment inserted in to the pALTER-1® vector (Promega). This synthetic C fragment gene encodes the same amino acids as does the naturally occurring C fragment gene. The naturally occurring C fragment sequences, like most clostridial genes, are extremely A/T rich (Thompson *et al., supra*). This high A/T content creates expression difficulties in *E. coli* and yeast due to altered codon usage frequency and fortuitous polyadenylation sites, respectively. In order to improve the expression of C fragment proteins in *E. coli*, a synthetic version of the gene was created in which the non-preferred codons were replaced with preferred codons.

The nucleotide sequence of the *C. hotulinum* C fragment gene sequences contained within pAlterBot is listed in SEQ ID NO:22. The first six nucleotides (ATGGCT) encode a methionine and alanine residue, respectively. These two amino acids result from the insertion of the *C. hotulinum* C fragment sequences into the pALTER® vector and provide the initiator methionine residue. The amino acid sequence of the *C. hotulinum* C fragment encoded by the sequences contained within pAlterBot is listed in SEQ ID NO:23. The first two amino acids (Met Ala) are encoded by vector-derived sequences. From the third amino acid residue onward (Arg), the amino acid sequence is identical to that found in the *C. hotulinum* type A toxin gene.

The pMA1870-2680, pPA1100-2680 and pAlterBot constructs were used as progenitor plasmids to make expression constructs in which fragments of the *C. difficile* toxin A repeat domain were expressed as genetic fusions with the *C. botulinum* C fragment gene using the pMAL-c expression vector (New England BioLabs). The pMAL-c expression vector generates fusion proteins which contain the MBP at the amino-terminal end of the protein. A construct, pMBot, in which the *C. botulinum* C fragment gene was expressed as a fusion with only the MBP was constructed (Figure 25). Fusion protein expression was induced from *E. coli* strains harboring the above plasmids, and induced protein was affinity purified on an amylose resin column.

i) C nstructi n Of pBlueBot

5

10

15

20

25

30

In order to facilitate the cloning of the C. hotulinum C fragment gene sequences into a number of desired constructs, the botulinal gene sequences were removed from pAlterBot and were inserted into the pBluescript plasmid (Stratagene) to generate pBlueBot (Figure 25). pBlueBot was constructed as follows. Bacteria containing the pAlterBot plasmid were grown in medium containing tetracycline and plasmid DNA was isolated using the QIAprep-spin Plasmid Kit (Qiagen). One microgram of pAlterBot DNA was digested with Neol and the resulting 3' recessed sticky end was made blunt using the Klenow fragment of DNA polymerase I (here after the Klenow fragment). The pAlterBot DNA was then digested with HindIII to release the botulinal gene sequences (the Bot insert) as a blunt (filled Neol site)-HindIII fragment. pBluescript vector DNA was prepared by digesting 200 ng of pBluescript DNA with Smal and HindIII. The digestion products from both plasmids were resolved on an agarose gel. The appropriate fragments were removed from the gel, mixed and purified utilizing the Prep-a-Gene kit (BioRad). The eluted DNA was then ligated using T4 DNA ligase and used to transform competent DH5\alpha cells (Gibco-BRL). Host cells were made competent for transformation using the calcium chloride protocol of Sambrook et al., supra at 1.82-1.83. Recombinant clones were isolated and confirmed by restriction digestion using standard recombinant molecular biology techniques (Sambrook et al. supra). The resultant clone, pBlueBot, contains several useful unique restriction sites flanking the Bot insert (i.e., the C. bottlinum C fragment sequences derived from pAlterBot) as shown in Figure 25.

ii) Construction Of C. difficile / C. botulinum / MBP Fusion Proteins

Constructs encoding fusions between the C. difficile toxin A gene and the C. botulinum C fragment gene and the MBP were made utilizing the same recombinant DNA methodology outlined above: these fusion proteins contained varying amounts of the C. difficile toxin A repeat domain.

The pMABot clone contains a 2.4 kb insert derived from the C. difficile toxin A gene fused to the Bot insert (i.e. the C. hotulinum C fragment sequences derived from pAlterBot). pMABot (Figure 25) was constructed by mixing gel-purified DNA from Notl/HindIII digested pBlueBot (the 1.2 kb Bot fragment). Spel/NotI digested pPA1100-2680 (the 2.4 kb C. difficile toxin A repeat fragment) and Xhal/HindIII digested pMAL-c vector. Recombinant clones were isolated, confirmed by restriction digestion and purified using the QIAprep-spin Plasmid

Kit (Qiagen). This clone expresses the toxin A repeats and the botulinal C fragment protein sequences as an in-frame fusion with the MBP.

The pMCABot construct contains a 1.0 kb insert derived from the C. difficile toxin A gene fused to the Bot insert (i.e. the C. botulinum C fragment sequences derived from pAlterBot). pMCABot was constructed by digesting the pMABot clone with EcoRI to remove the 5' end of the C. difficile toxin A repeat (see Figure 25, the pMAL-c vector contains a EcoRI site 5' to the C. difficile insert in the pMABot clone). The restriction sites were filled and religated together after gel purification. The resultant clone (pMCABot. Figure 25) generated an in-frame fusion between the MBP and the remaining 3' portion of the C. difficile toxin A repeat domain fused to the Bot gene.

. 5 .

10

15

20

25

30

The pMNABot clone contains the 1 kb Spel/EcoRI (filled) fragment from the C. difficile toxin A repeat domain (derived from clone pPA1100-2680) and the 1.2 kb C. botulinum C fragment gene as a Ncol (filled)/HindIII fragment (derived from pAlterBot). These two fragments were inserted into the pMAL-c vector digested with Nbal/HindIII. The two insert fragments were generated by digestion of the appropriate plasmid with EcoRI (pPA1100-2680) or Ncol (pAlterBot) followed by treatment with the Klenow fragment. After treatment with the Klenow fragment, the plasmids were digested with the second enzyme (either Spel or HindIII). All three fragments were gel purified, mixed and Prep-a-Gene purified prior to ligation. Following ligation and transformation, putative recombinants were analyzed by restriction analysis: the EcoRI site was found to be regenerated at the fusion junction, as was predicted for a fusion between the filled EcoRI and Ncol sites.

A construct encoding a fusion protein between the botulinal C fragment gene and the MBP gene was constructed (i.e., this fusion lacks any C difficile toxin A gene sequences) and termed pMBot. The pMBot construct was made by removal of the C difficile toxin A sequences from the pMABot construct and fusing the C fragment gene sequences to the MBP. This was accomplished by digestion of pMABot DNA with Stul (located in the pMALc polylinker 5° to the Xhal site) and Xhal (located 3° to the Notl site at the toxA-Bot fusion junction), filling in the Xhal site using the Klenow fragment, gel purifying the desired restriction fragment, and ligating the blunt ends to circularize the plasmid. Following ligation and transformation, putative recombinants were analyzed by restriction mapping of the Bot insert (i.e., the C botulinum C fragment sequences).

b) Expression Of C. botulinum C Fragment Fusion Pr teins In E. coli

5

10

15

20

25

Large scale (1 liter) cultures of the pMAL-c vector, and each recombinant construct described above in (a) were grown, induced, and soluble protein fractions were isolated as described in Example 18. The soluble protein extracts were chromatographed on amylose affinity columns to isolate recombinant fusion protein. The purified recombinant fusion proteins were analyzed by running samples on SDS-PAGE gels followed by Coomassic staining and by Western blot analysis as described [Williams *et al.* (1994) *supra*]. In brief, extracts were prepared and chromatographed in column buffer (10 mM NaPO₄, 0.5 M NaCl, 10 mM β-mercaptoethanol, pH 7.2) over an amylose resin (New England Biolabs) column, and eluted with column buffer containing 10 mM maltose as described [Williams, *et al.* (1994), *supra*]. An SDS-PAGE gel containing the purified protein samples stained with Coomassie blue is shown in Figure 26.

In Figure 26, the following samples were loaded. Lanes 1-6 contain protein purified from *E. coli* containing the pMAL-c, pPA1870-2680, pMABot, pMNABot, pMCABot and pMBot plasmids, respectively. Lane 7 contains broad range molecular weight protein markers (BioRad).

The protein samples were prepared for electrophoresis by mixing 5 μl of eluted protein with 5 μl of 2X SDS-PAGE sample buffer (0.125 mM Tris-HCl, pH 6.8, 2 mM EDTA, 6% SDS, 20% glycerol, 0.025% bromophenol blue: β-mercaptoethanol is added to 5% before use). The samples were heated to 95°C for 5 min, then cooled and loaded on a 7.5% agarose SDS-PAGE gel. Broad range molecular weight protein markers were also loaded to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected generally by staining the gel with Coomassie blue.

In all cases the yields were in excess of 20 mg fusion protein per liter culture (see Table 36) and, with the exception of the pMCABot protein, a high percentage (i.e., greater than 20-50% of total cluted protein) of the cluted fusion protein was of a MW predicted for the full length fusion protein (Figure 26). It was estimated (by visual inspection) that less than 10% of the pMCABot fusion protein was expressed as the full length fusion protein.

TABLE 36

Yield Of Affinity Purified C. hotulinum C Fragment / MBP Fusion Proteins

Construct	Yield (mg/liter of Culture)	Percentage ()f Total Soluble Protein		
рМАВоі	24	5.0		
pMCABot	34	5.0		
pMNABot	40	5.5		
pMBot	22	5.0		
pMA1870-2680	40	4.8		

5

10

15

20

25

30

These results demonstrate that high level expression of intact C. botulinum C fragment/C. difficile toxin A fusion proteins in E. coli is feasible using the pMAL-c expression system. These results are in contrast to those reported by H. F. LaPenotiere, et al. (1993), supra. In addition, these results show that it is not necessary to fuse the botulinal C fragment gene to the C. difficile toxin A gene in order to produce a soluble fusion protein using the pMAL-c system in E. coli.

In order to determine whether the above-described botulinal fusion proteins were recognized by anti-C. botulinum toxin A antibodies. Western blots were performed. Samples containing affinity-purified proteins from E. coli containing the pMABot, pMCABot, pMNABot, pMBot, pMA1870-2680 or pMALc plasmids were analyzed. SDS-PAGE gels (7.5% acrylamide) were loaded with protein samples purified from each expression construct. After electrophoresis, the gels were blotted and protein transfer was confirmed by Ponceau S staining (as described in Example 12b).

Following protein transfer, the blots were blocked by incubation for 1 hr at 20°C in blocking buffer [PBST (PBS containing 0.1% Tween 20 and 5% dry milk)]. The blots were then incubated in 10 ml of a solution containing the primary antibody: this solution comprised a 1/500 dilution of an anti-*C. botulinum* toxin A IgY PEG prep (described in Example 3) in blocking buffer. The blots were incubated for 1 hr at room temperature in the presence of the primary antibody. The blots were washed and developed using a rabbit anti-chicken alkaline phosphatase conjugate (Bochringer Mannheim) as the secondary antibody as follows. The rabbit anti-chicken antibody was diluted to 1 µg/ml in blocking buffer (10 ml final volume per blot) and the blots were incubated at room temperature for 1 hour in the presence of the secondary antibody. The blots were then washed successively with PBST. BBS-Tween and 50 mM Na₂CO₃, pH 9.5. The blots were then developed in freshly-prepared alkaline

phosphatase substrate buffer (100 μ g/ml nitro blue tetrazolium, 50 μ g/ml 5-bromo-chloro-indolylphosphate, 5 mM MgCl₂ in 50 mM Na₂CO₃, pH 9.5). Development was stopped by flooding the blots with distilled water and the blots were air dried.

This Western blot analysis detected anti-C. botulinum toxin reactive proteins in the pMABot, pMCABot, pMNABot and pMBot protein samples (corresponding to the predicted full length proteins identified above by Coomassie staining in Figure 26), but not in the pMA1100-2680 or pMALe protein samples.

These results demonstrate that the relevant fusion proteins purified on an amylose resin as described above in section a) contained immunoreactive *C botulinum C* fragment protein as predicted.

EXAMPLE 23

Generation Of Neutralizing Antibodies

By Nasal Administration Of pMBot Protein

15

10

5

The ability of the recombinant botulinal toxin proteins produced in Example 22 to stimulate a systemic immune response against botulinal toxin epitopes was assessed. This example involved: a) the evaluation of the induction of serum IgG titers produced by nasal or oral administration of botulinal toxin-containing C. difficile toxin A fusion proteins and b) the *in vivo* neutralization of C. botulinum type A neurotoxin by anti- recombinant C. botulinum C fragment antibodies.

Evaluation Of The Induction Of Serum IgG Titers Produced
 By Nasal Or Oral Administration Of Botulinal Toxin Containing C. difficile Toxin A Fusion Proteins

25

30

20

Six groups containing five 6 week old CF female rats (Charles River) per group were immunized nasally or orally with one of the following three combinations using protein prepared in Example 22: (1) 250 μg pMBot protein per rat (nasal and oral): 2) 250 μg pMABot protein per rat (nasal and oral): 3) 125 μg pMBot admixed with 125 μg pMA1870-2680 per rat (nasal and oral). A second set of 5 groups containing 3 CF female rats/group were immunized nasally or orally with one of the following combinations (4) 250 μg pMNABot protein per rat (nasal and oral) or 5) 250 μg pMAL-c protein per rat (nasal and oral).

The fusion proteins were prepared for immunization as follows. The proteins (in column buffer containing 10 mM maltose) were diluted in 0.1 M carbonate buffer, pH 9.5 and administered orally or nasally in a 200 µl volume. The rats were lightly sedated with ether prior to administration. The oral dosing was accomplished using a 20 gauge feeding needle. The nasal dosing was performed using a P-200 micro-pipettor (Gilson). The rats were boosted 14 days after the primary immunization using the techniques described above and were bled 7 days later. Rats from each group were lightly etherized and bled from the tail. The blood was allowed to clot at 37°C for 1 hr and the serum was collected.

. 5

10

15

20

25

30

The serum from individual rats was analyzed using an ELISA to determine the anti-C. botulinum type A toxin IgG serum titer. The ELISA protocol used is a modification of that described in Example 13c. Briefly, 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were coated with C. botulinum type A toxoid (prepared as described in Example 3a) by placing 100 µl volumes of C. botulinum type A toxoid at 2.5 µg/ml in PBS containing 0.005% thimerosal in each well and incubating overnight at 4°C. The next morning, the coating suspensions were decanted and all wells were washed three times using PBS.

In order to block non-specific binding sites, 100 µl of blocking solution [0.5% BSA in PBS] was then added to each well and the plates were incubated for 1 hr at 37°C. The blocking solution was decanted and duplicate samples of 150 µl of diluted rat serum added to the first well of a dilution series. The initial testing serum dilution was 1:30 in blocking solution containing 0.5% Tween 20 followed by 5-fold dilutions into this solution. This was accomplished by serially transferring 30 µl aliquots to 120 µl blocking solution containing 0.5% Tween 20, mixing, and repeating the dilution into a fresh well. After the final dilution, 30 µl was removed from the well such that all wells contained 120 µl final volume. A total of 3 such dilutions were performed (4 wells total). The plates were incubated 1 hr at 37°C. Following this incubation, the serially diluted samples were decanted and the wells were washed six times using PBS containing 0.5% Tween 20 (PBST). To each well, 100 µl of a rabbit anti-Rat IgG alkaline phosphatase (Sigma) diluted (1/1000) in blocking buffer containing 0.5% Tween 20 was added and the plate was incubated for 1 hr at 37°C. The conjugate solutions were decanted and the plates were washed as described above, substituting 50 mM Na₂CO₃, pH 9.5 for the PBST in the final wash. The plates were developed by the addition of 100 µl of a solution containing 1 mg/ml para-nitro phenyl phosphate (Sigma) dissolved in 50 mM Na₂CO₃, 10 mM MgCl₂, pH 9.5 to each well, and incubating the plates at room temperature in the dark for 5-45 min. The absorbency of each well was measured at

410 nm using a Dynatech MR 700 plate reader. The results are summarized in Tables 37 and 38 and represent mean serum reactivities of individual mice.

Determination Of Anti-C bondinum Type A Toxin Serum IgG Titers Following Immunization With C. bondinum C Fragment-Containing Fusion Proteins

5

10

15

20

25

30

35

Route of Immunization			Nasal		Oral			
lmmunogen	PRE- IMMUNF	pMBot	pMBot & pMA1870- 2680	pMABot	рМВы	рМВоке рМА1870- 2680	pMAthii	
Dilution								
1.30	0.080	1 040	1 030	0 060	0.190	080.0	0.120	
1 150	0.017	0.580	0.540	0.022	0.070	0.020	0.027	
1.750	0.009	0.280	0.260	0.010	0 020	0.010	0.014	
1:3750	0.007	0 084	0.090	0 009	0 009	0.010	0.007	
# Rats Tested		5	•	5	;	2	<u>;</u>	

Numbers represent the average values obtained from two ELISA plates, standardized utilizing the preimmane control

TABLE 38

Determination Of Anti-C. borulinum Type A Toxin Serum IgG Titers
Following Immunization With C. borulinum C Fragment-Containing Fusion Proteins

Route of Ir	nmunization	N	nsal	Oral		
Immunogen	PRE-IMMUNE	pMBot	рМАВог	pMNABot	pMNABoi	
Dilution						
1:30	0.040	0.557	0.010	0.015	0.010	
1:150	0.009	0.383	0.001	0.003	0.002	
1:750	0.00}	0.140	0.000	0.000	0.000	
1:3750	0.000	0.040	0.000	0.000	0.000	
# Rats Tested		1	1	3	3	

The above ELISA results demonstrate that reactivity against the botulinal fusion proteins was strongest when the route of administration was nasal; only weak responses were stimulated when the botulinal fusion proteins were given orally. Nasally delivered pMbot and pMBot admixed with pMA1870-2680 invoked the greatest serum IgG response. These results show that only the pMBot protein is necessary to induce this response, since the addition of the pMA1870-2680 protein did not enhance antibody response (Table 37). Placement of the C. difficile toxin A fragment between the MBP and the C. botulinum C fragment protein

- 150 -

dramatically reduced anti-bot IgG titer (see results using pMABot, pMCABot and pMNABot proteins).

This study demonstrates that the pMBot protein induces a strong serum IgG response directed against C. hotulinum type A toxin when nasally administered.

5

10

15

b) In Vivo Neutralization Of C. botulinum Type A Neurotoxin By Anti- Recombinant C. botulinum C Fragment Antibodies

The ability of the anti-C. botulinum type A toxin antibodies generated by nasal administration of recombinant botulinal fusion proteins in rats (Example 22) to neutralize C. botulinum type A toxin was tested in a mouse neutralization model. The mouse model is the art accepted method for detection of botulinal toxins in body fluids and for the evaluation of anti-botulinal antibodies [E.J. Schantz and D.A. Kautter, J. Assoc. Off. Anal. Chem. 61:96 (1990) and Investigational New Drug (BB-IND-3703) application by the Surgeon General of the Department of the Army to the Federal Food and Drug Administration]. The anti-C. botulinum type A toxin antibodies were prepared as follows.

Rats from the group given pMBot protein by nasal administration were boosted a second time with 250 µg pMBot protein per rat and serum was collected 7 days later. Serum from one rat from this group and from a preimmune rat was tested for anti-C. botulinum type A toxin neutralizing activity in the mouse neutralization model described below.

20

25

30

The LD_{so} of a solution of purified *C. botulinum* type A toxin complex, obtained from Dr. Eric Johnson (University of Wisconsin Madison), was determined using the intraperitoneal (IP) method of Schantz and Kautter [J. Assoc. Off. Anal. Chem. 61:96 (1978)] using 18-22 gram female ICR mice and was found to be 3500 LD_{so}/ml. The determination of the LD_{so} was performed as follows. A Type A toxin standard was prepared by dissolving purified type A toxin complex in 25 mM sodium phosphate buffer, pH 6.8 to yield a stock toxin solution of 3.15 x 10⁷ LD_{so}/mg. The OD₂₇₈ of the solution was determined and the concentration was adjusted to 10-20 µg/ml. The toxin solution was then diluted 1:100 in gel-phosphate (30 mM phosphate, pH 6.4; 0.2% gelatin). Further dilutions of the toxin solution were made as shown below in Table 39. Two mice were injected IP with 0.5 ml of each dilution shown and the mice were observed for symptoms of botulism for a period of 72 hours.

- 151 -

TABLE 39

Determination Of The LD_{so} Of Purified C. hotulinum Type A Toxin Complex

Dilution	Number Dead At 72 hr				
1:320	2/2				
1:640	2/2				
1:1280	2/2				
1:2560	0/2 (sick after 72 hr)				
1:5120	0/2 (no symptoms)				

5

10

15

20

25

30

From the results shown in Table 39, the toxin titer was assumed to be between 2560 LD_{so}/ml and 5120 LD_{so}/ml (or about 3840 LD_{so}/ml). This value was rounded to 3500 LD_{so}/ml for the sake of calculation.

The amount of neutralizing antibodies present in the serum of rats immunized nasally with pMBot protein was then determined. Serum from two rats boosted with pMBot protein as described above and preimmune serum from one rat was tested as follows. The toxin standard was diluted 1:100 in gel-phosphate to a final concentration of 350 LD_{so}/ml. One milliliter of the diluted toxin standard was mixed with 25 µl of serum from each of the three rats and 0.2 ml of gel-phosphate. The mixtures were incubated at room temperature for 30 min with occasional mixing. Each of two mice were injected with IP with 0.5 ml of the mixtures. The mice were observed for signs of botulism for 72 hr. Mice receiving serum from rats immunized with pMBot protein neutralized this challenge dose. Mice receiving preimmune rat serum died in less than 24 hr.

The amount of neutralizing anti-toxin antibodies present in the serum of rats immunized with pMBot protein was then quantitated. Serum antibody titrations were performed by mixing 0.1 ml of each of the antibody dilutions (see Table 40) with 0.1 ml of a 1:10 dilution of stock toxin solution (3.5 x 10⁴ LD₅₀/ml) with 1.0 ml of gel-phosphate and injecting 0.5 ml IP into 2 mice per dilution. The mice were then observed for signs of botulism for 3 days (72 hr). The results are tabulated in Table 39.

As shown in Table 40 pMBot serum neutralized *C. hotulinum* type A toxin complex when used at a dilution of 1:320 or less. A mean neutralizing value of 168 IU/ml was obtained for the pMBot serum (an IU is defined as 10.000 mouse LD_{so}). This value translates to a circulating serum titer of about 3.7 IU/mg of serum protein. This neutralizing titer is comparable to the commercially available bottled concentrated (Connaught Laboratories, Ltd.) horse anti-*C. hotulinum* antiserum. A 10 ml vial of Connaught antiserum contains about 200

mg/ml of protein:each ml can neutralize 750 IU of *C. hotulinum* type A toxin. After administration of one vial to a human, the circulating serum titer of the Connaught preparation would be approximately 25 IU/ml assuming an average serum volume of 3 liters). Thus, the circulating anti-*C. hotulinum* titer seen in rats nasally immunized with pMBot protein (168 IU/ml) is 6.7 time higher than the necessary circulation titer of anti-*C. hotulinum* antibody needed to be protective in humans.

TABLE 40

Quantitation Of Neutralizing Antibodies In pMBot Sera

Dilution	pMBat*				
	Rat I	Rat 2			
1:20	2/2	2/2			
1:40	2/2	2/2			
1:80	2/2	2.5			
1:160	2.2	2/2			
1:320	2/2h	2/2 ^b			
1:640	0/2	0/2			
1:1280	0/2	0/2			
1:2560	0/2	0/2			

Numbers represent the number of mice surviving at 72 hours which received serum taken from rats immunized with the pMBot protein.

These mice survived but were sick after 72 hr.

These results demonstrate that antibodies capable of neutralizing C, botulinum type A toxin are induced when recombinant C, botulinum C fragment fusion protein produced in E, coli is used as an immunogen.

EXAMPLE 24

Production Of Soluble C. botulinum C Fragment
Protein Substantially Free Of Endotoxin Contamination

30

5

10

15

20

25

Example 23 demonstrated that neutralizing antibodies are generated by immunization with the pMBot protein expressed in *E. coli*. These results showed that the pMBot fusion protein is a good vaccine candidate. However, immunogens suitable for use as vaccines should be pyrogen-free in addition to having the capability of inducing neutralizing

antibodies. Expression clones and conditions that facilitate the production of *C. hotulinum* C fragment protein for utililization as a vaccine were developed.

The example involved: (a) determination of pyrogen content of the pMBot protein: (b) generation of C. botulinum C fragment protein free of the MBP: (c) expression of C. botulinum C fragment protein using various expression vectors: and (d) purification of soluble C. botulinum C fragment protein substantially free of significant endotoxin contamination.

a) Determination Of The Pyrogen Content Of The pMBot Protein

5

10

15

20

25

30

In order to use a recombinant antigen as a vaccine in humans or other animals, the antigen preparation must be shown to be free of pyrogens. The most significant pyrogen present in preparations of recombinant proteins produced in gram-negative bacteria, such as *E. coli.* is endotoxin [F.C. Pearson, *Pyrogens: endotoxins, LAL testing and depyrogentation*, (1985) Marcel Dekker, New York, pp. 23-56]. To evaluate the utility of the pMBot protein as a vaccine candidate, the endotoxin content in MBP fusion proteins was determined.

The endotoxin content of recombinant protein samples was assayed utilizing the Limulus assay (LAL kit: Associates of Cape Cod) according to the manufacturer's instructions. Samples of affinity-purified pMal-c protein and pMA1870-2680 were found to contain high levels of endotoxin [>50.000 EU/mg protein: EU (endotoxin unit)]. This suggested that MBP- or toxin A repeat-containing fusions with the botulinal C fragment should also contain high levels of endotoxin. Accordingly, removal of endotoxin from affinity-purified pMal-c and pMBot protein preparations was attempted as follows.

Samples of pMal-c and pMBot protein were depyrogenated with polymyxin to determine if the endotoxin could be easily removed. The following amount of protein was treated: 29 ml at 4.8 OD₂₈₀/ml for pMal-c and 19 mls at 1.44 OD₂₈₀/ml for pMBot. The protein samples were dialyzed extensively against PBS and mixed in a 50 ml tube (Falcon) with 0.5 ml PBS-equilibrated polymyxin B (Affi-Prep Polymyxin. BioRad). The samples were allowed to mix by rotating the tubes overnight at 4°C. The polymyxin was pelleted by centrifugation for 30 min in a bench top centrifuge at maximum speed (approximately 2000 x g) and the supernatant was removed. The recovered protein (in the supernatant) was quantified by OD₂₈₀, and the endotoxin activity was assayed by LAL. In both cases only approximately 1/3 of the input protein was recovered and the polymyxin-treated protein retained significant endotoxin contamination (approximately 7000 EU/mg of pMBot).

The depyrogenation experiment was repeated using an independently purified pMal-c protein preparation and similar results were obtained. From these studies it was concluded that significant levels of endotoxin copurifies with these MBP fusion proteins using the amylose resin. Furthermore, this endotoxin cannot be easily removed by polymyxin treatment.

These results suggest that the presence of the MBP sequences on the fusion protein complicated the removal of endotoxin from preparations of the pMBot protein.

b) Generation Of C. botulinum C Fragment Protein Free Of The MBP

5

10

15

20

25

30

It was demonstrated that the pMBot fusion protein could not be easily purified from contaminating endotoxin in section a) above. The ability to produce a pyrogen-free (e.g., endotoxin-free) preparation of soluble botulinal C fragment protein free of the MBP tag was next investigated. The pMBot expression construct was designed to facilitate purification of the botulinal C fragment from the MBP tag by cleavage of the fusion protein by utilizing an engineered Factor Xa cleavage site present between the MBP and the botulinal C fragment. The Factor Xa cleavage was performed as follows.

Factor Xa (New England Biolabs) was added to the pMBot protein (using a 0.1-1.0% Factor Xa/pMBot protein ratio) in a variety of buffer conditions [e.g., PBS-NaCl (PBS containing 0.5 M NaCl), PBS-NaCl containing 0.2% Tween 20, PBS, PBS containing 0.2% Tween 20, PBS-C (PBS containing 2 mM CaCl₂), PBS-C containing either 0.1 or 0.5 % Tween 20, PBS-C containing either 0.1 or 0.5% NP-40, PBS-C containing either 0.1 or 0.5% Triton X-100, PBS-C containing 0.1% sodium deoxycholate, PBS-C containing 0.1% SDS]. The Factor Xa digestions were incubated for 12-72 hrs at room temperature.

The extent of cleavage was assessed by Western blot or Coomassie blue staining of proteins following electrophoresis on denaturing SDS-PAGE gels, as described in Example 22. Cleavage reactions (and control samples of uncleaved pMBot protein) were centrifuged for 2 min in a microfuge to remove insoluble protein prior to loading the samples on the gel. The Factor Xa treated samples were compared with uncleaved, uncentrifuged pMBot samples on the same gel. The results of this analysis is summarized below.

1) Most (about 90%) pMBot protein could be removed by centrifugation, even when uncleaved control samples were utilized. This indicated that the pMBot fusion protein was not fully soluble (i.e., it exists as a suspension rather than as a solution). [This result was

consistent with the observation that most affinity-purified pMBot protein precipitates after long term storage (>2 weeks) at 4°C. Additionally, the majority (i.e., 75%) of induced pMBot protein remains in the pellet after sonication and clarification of the induced E. coli. Resuspension of these insoluble pellets in PBS followed by sonication results in partial solubilization of the insoluble pMBot protein in the pellets.

5

10

15

20

25

30

- 2) The portion of pMBot protein that is fully in solution (about 10% of pMBot protein) is completely cleaved by Factor Xa, but the cleaved (released) botulinal C fragment is relatively insoluble such that only the cleaved MBP remains fully in solution.
- 3) None of the above reaction conditions enhanced solubility without also reducing effective cleavage. Conditions that effectively solubilized the cleaved botulinal C fragment were not identified.
- 4) The use of 0.1% SDS in the buffer used for Factor Xa cleavage enhanced the solubility of the pMBot protein (all of pMBot protein was soluble). However, the presence of the SDS prevented any cleavage of the fusion protein with Factor Xa.
- 5) Analysis of pelleted protein from the cleavage reactions indicated that both full length pMBot (i.e., uncleaved) and cleaved botulinal C fragment protein precipitated during incubation.

These results demonstrate that purification of soluble botulinal C fragment protein after cleavage of the pMBot fusion protein is complicated by the insolubility of both the pMBot protein and the cleaved botulinal C fragment protein.

c) Expression Of C. botulinum C Fragment Using Various Expression Vectors

In order to determine if the solubility of the botulinal C fragment was enhanced by expressing the C fragment protein as a native protein, an N-terminal His-tagged protein or as a fusion with glutathione-S-transferase (GST), alternative expression plasmids were constructed. These expression constructs were generated utilizing the methodologies described in Example 22. Figure 27 provides a schematic representation of the vectors described below.

In Figure 27, the following abbreviations are used. pP refers to the pET23 vector. pHIS refers to the pETHisa vector. pBlue refers to the pBluescript vector. pM refers to the pMAL-c vector and pG refers to the pGEX3T vector (described in Example 11). The solid black lines represent *C. botulinum* C fragment gene sequences: the solid black ovals represent the MBP: the hatched ovals represent GST; "HHHHHH" represents the poly-histidine tag. In

Figure 27, when the name for a restriction enzyme appears inside parenthesis, this indicates that the restriction site was destroyed during construction. An asterisk appearing with the name for a restriction enzyme indicates that this restriction site was recreated at a cloning junction.

1.5

10

15

20

25

30

i) Construction Of pPBot

In order to express the *C. botulinum* C fragment as a native (*i.e.*, non-fused) protein, the pPBot plasmid (shown schematically in Figure 27) was constructed as follows. The C fragment sequences present in pAlterBot (Example 22) were removed by digestion of pAlterBot with *Ncol* and *Hind*III. The *Ncol/Hind*III C fragment insert was ligated to pETHisa vector (described in Example 18b) which was digested with *Ncol* and *Hind*III. This ligation creates an expression construct in which the *Ncol*-encoded methionine of the botulinal C fragment is the initiator codon and directs expression of the native botulinal C fragment. The ligation products were used to transform competent BL21(DE3)pLysS cells (Novagen). Recombinant clones were identified by restriction mapping.

ii) Construction Of pHisBot

In order to express the *C. hotulinum* C fragment containing a poly-histidine tag at the amino-terminus of the recombinant protein, the pHisBot plasmid (shown schematically in Figure 27) was constructed as follows. The *Ncol/Hind*III botulinal C fragment insert from pAlterbot was ligated into the pETHisa vector which was digested with *Nhel* and *Hind*III. The *Ncol* (on the C fragment insert) and *Nhel* (on the pETHisa vector) sites were filled in using the Klenow fragment prior to ligation; these sites were then blunt end ligated (the *Ndel* site was regenerated at the clone junction as predicted). The ligation products were used to transform competent BL21(DE3)pLysS cells and recombinant clones were identified by restriction mapping.

The resulting pHisBot clone expresses the botulinal C fragment protein with a histidine-tagged N-terminal extension having the following sequence: MetGlyHisHis HisHisHisHisHisHisHisHisHisHeGluGlyArgHisMetAla. (SEQ ID NO:24): the amino acids encoded by the botulinal C fragment gene are underlined and the vector encoded amino acids are presented in plain type. The nucleotide sequence present in the pETHisa vector which encodes the pHisBot fusion protein is listed in SEQ ID NO:25. The amino acid sequence of the pHisBot protein is listed in SEQ ID NO:26.

iii) C nstruction Of pGBot

The botulinal C fragment protein was expressed as a fusion with the glutathione-S-transferase protein by constructing the pGBot plasmid (shown schematically in Figure 27). This expression construct was created by cloning the Notl/Sall C fragment insert present in pBlueBot (Example 22) into the pGEX3T vector which was digested with Smal and Xhol. The Notl site (present on the botulinal fragment) was made blunt prior to ligation using the Klenow fragment. The ligation products were used to transform competent BL21 cells.

Each of the above expression constructs were tested by restriction digestion to confirm the integrity of the constructs.

10

5

Large scale (1 liter) cultures of pPBot [BL21(DE3)pLysS host], pHisBot [BL21(DE3)pLysS host] and pGBot (BL21 host) were grown in 2X YT medium and induced (using IPTG to 0.8-1.0 mM) for 3 hrs as described in Example 22. Total, soluble and insoluble protein preparations were prepared from 1 ml aliquots of each large scale culture [Williams et al. (1994), supra] and analyzed by SDS-PAGE. No obvious induced band was detectable in the pPBot or pHisBot samples by Coomassie staining, while a prominent insoluble band of the anticipated MW was detected in the pGBot sample. Soluble lysates of the pGBot large scale (resuspended in PBS) or pHisBot large scale [resuspended in Novagen 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9)] cultures were prepared and used to affinity purify soluble affinity-tagged protein as follows.

20

15

The pGBot lysate was affinity purified on a glutathione-agarose resin (Pharmacia) exactly as described in Smith and Corcoran [Current Protocols in Molecular Biology, Supplement 28 (1994), pp. 16.7.1-16.7.7]. The pHisBot protein was purified on the His-Bind resin (Novagen) utilizing the His-bind buffer kit (Novagen) exactly as described by manufacturer.

25

Samples from the purification of both the pGBot and pHisBot proteins (including uninduced, induced, total, soluble, and affinity-purified cluted protein) were resolved on SDS-PAGE gels. Following electrophoresis, proteins were analyzed by Coomassie staining or by Western blot detection utilizing a chicken anti-C. botulinum Type A toxoid antibody (as described in Example 22).

30

These studies showed that the pGBot protein was almost entirely insoluble under the utilized conditions, while the pHisBot protein was soluble. Affinity purification of the pHisBot protein on this first attempt was inefficient, both in terms of yield (most of the

immunoreactive botulinal protein did not bind to the His-bind resin) and purity (the botulinal protein was estimated to comprise approximately 20% of the total eluted protein).

d) Purification Of Soluble C. botulinum C Fragment Protein Substantially Free Of Endotoxin Contamination

• 5 •

10

15

20

25

30

The above studies showed that the pHisBot protein was expressed in $E.\ coli$ as a soluble protein. However, the affinity purification of this protein on the His-bind resin was very inefficient. In order to improve the affinity purification of the soluble pHisBot protein (in terms of both yield and purity), an alternative poly-histidine binding affinity resin (Ni-NTA resin: Qiagen) was utilized. The Ni-NTA resin was reported to have a superior binding affinity ($K_d=1 \times 10^{-13}$ at pH 8.0: Qiagen user manual) relative to the His-bind resin.

A soluble lysate (in Novagen 1X binding buffer) from an induced 1 liter 2X YT culture was prepared as described above. Briefly, the culture of pHisBot [BI21(DE3)pLysS host] was grown at 37°C to an OD₆₀₀ of 0.7 in 1 liter of 2X YT medium containing 100 μg/ml ampicillin, 34 μg/ml chloramphenicol and 0.2% glucose. Protein expression was induced by the addition of IPTG to 1 mM. Three hours after the addition of the IPTG, the cells were cooled for 15 min in a ice water bath and then centrifuged 10 min at 5000 rpm in a JA10 rotor (Beckman) at 4°C. The pellets were resuspended in a total volume of 40 mls Novagen 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), transferred to two 35 ml Oakridge tubes and frozen at -70°C for at least 1 hr. The tubes were thawed and the cells were lysed by sonication (4 X 20 second bursts using a Branson Sonifier 450 with a power setting of 6-7) on ice. The suspension was clarified by centrifugation for 20 min at 9,000 rpm (10,000 x g) in a JA-17 rotor (Beckman).

The soluble lysate was brought to 0.1% NP40 and then was batch absorbed to 7 ml of a 1:1 slurry of Ni-NTA resin:binding buffer by stirring for 1 hr at 4°C. The slurry was poured into a column having an internal diameter of 1 or 2.5 cm (BioRad). The column was then washed sequentially with 15 mls of Novagen 1X binding buffer containing 0.1% NP40. 15 ml of Novagen 1X binding buffer, 15 ml wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pl4 7.9) and 15 ml NaHPO₄ wash buffer (50 mM NaHPO₄, pl4 7.0, 0.3 M NaCl, 10 % glycerol). The bound protein was eluted by protonation of the resin using elution buffer (50 mM NaHPO₄, pH 4.0, 0.3 M NaCl, 10 % glycerol). The cluted protein was stored at 4°C.

Samples of total, soluble and eluted protein were resolved by SDS-PAGE. Protein samples were prepared for electrophoresis as described in Example 22b. Duplicate gels were stained with Coomassie blue to visualize the resolved proteins and *C. botulinum* type A toxin-reactive protein was detected by Western blot analysis as described in Example 22b. A representative Coomassie stained gel is shown in Figure 28. In Figure 28, the following samples were loaded on the 12.5% acrylamide gel. Lanes 1-4 contain respectively total protein, soluble protein, soluble protein present in the flow-through of the Ni-NTA column and affinity-purified pHisBot protein (*i.e.*, protein released from the Ni-NTA resin by protonation). Lane 5 contains high molecular weight protein markers (BioRad).

10

5

The purification of pHisBot protein resulted in a yield of 7 mg of affinity purified protein from a 1 liter starting culture of BL21(DE3)pLysS cells harboring the pHisBot plasmid. The yield of purified pHisBot protein represented approximately 0.4% of the total soluble protein in the induced culture. Analysis of the purified pHisBot protein by SDS-PAGE revealed that at least 90-95% of the protein was present as a single band (Figure 28) of the predicted MW (50 kD). This 50 kD protein band was immunoreactive with anti-C botulinum type A toxin antibodies. The extinction coefficient of the protein preparation was determined to be 1.4 (using the Pierce BCA assay) or 1.45 (using the Lowry assay) OD₃₈₀ per 1 mg/ml solution.

20

15

Samples of pH neutralized eluted pHisBot protein were resolved on a KB 803 HPLC column (Shodex). Although His-tagged proteins are retained by this sizing column (perhaps due to the inherent metal binding ability of the proteins), the relative mobility of the pHisBot protein was consistent with that expected for a non-aggregated protein in solution. Most of the induced pHisBot protein was determined to be soluble under the growth and solubilization conditions utilized above (i.e., greater than 90% of the pHisBot protein was found to be soluble as judged by comparison of the levels of pHisBot protein seen in total and soluble protein samples prepared from BL21(DE3)pLysS cells containing the pHisBot plasmid). SDS-PAGE analysis of samples obtained after centrifugation, extended storage at -20°C, and at least 2 cycles of freezing and thawing detected no protein loss (due to precipitation), indicating that the pHisBot protein is soluble in the elution buffer (i.e., 50 mM NaHPO), pH 4.0, 0.3 M NaCl, 10 % glycerol).

30

25

Determination of endotoxin contamination in the affinity purified pHisBot preparation (after pH neutralization) using the LAL assay (Associates of Cape Cod) detected no significant endotoxin contamination. The assay was performed using the endpoint

chromogenic method (without diazo-coupling) according to the manufacturer's instructions. This method can detect concentrations of endotoxin greater than or equal to 0.03 EU/ml (EU refers to endotoxin units). The LAL assay was run using 0.5 ml of a solution comprising 0.5 mg pHisBot protein in 50 mM NaHPO₄, pH 7.0, 0.3 M NaCl, 10 % glycerol; 30-60 EU were detected in the 0.5 ml sample. Therefore, the affinity purified pHisBot preparation contains 60-120 EU/mg of protein. FDA Guidelines for the administration of parenteral drugs require that a composition to be administered to a human contain less than 5 EU/kg body weight (The average human body weight is 70 kg; therefore up to 349 EU units can be delivered in a parental dose.). Because very small amount of protein are administered in a vaccine preparation (generally in the range of 10-500 µg of protein), administration of affinity purified pHisBot containing 60-120 EU/mg protein would result in delivery of only a small percentage of the permissible endotoxin load. For example, administration of 10-500 µg of purified pHisBot to a 70 kg human, where the protein preparation contains 60 EU/mg protein, results in the introduction of only 0.6 to 30 EU [i.e., 0.2 to 8.6% of the maximum allowable endotoxin burden per parenteral dose (less than 5 EU/kg body weight)].

The above results demonstrate that endotoxin (LPS) does not copurify with the pHisBot protein using the above purification scheme. Preparations of recombinantly produced pHisBot protein containing lower levels of endotoxin (less than or equal to 2 EU/ mg recombinant protein) may be produced by washing the Ni-NTA column with wash buffer until the OD₃₈₀ returns to baseline levels (*i.e.*, until no more UV-absorbing material comes off of the column).

The above results illustrate a method for the production and purification of soluble, botulinal C fragment protein substantially free of endotoxin.

25

30

. 5

10

15

20

EXAMPLE 25

Optimization Of The Expression And Purification Of pHisBot Protein

The results shown in Example 24d demonstrated that the pHisBot protein is an excellent candidate for use as a vaccine as it could be produced as a soluble protein in *E. coli* and could be purified free of pyrogen activity. In order to optimize the expression and purification of the pHisBot protein, a variety of growth and purification conditions were tested.

a) Growth Parameters

5

10

15

20

25

30

i) Host Strains

The influence of the host strain utilized upon the production of soluble pHisBot protein was investigated. A large scale purification of pHisBot was performed [as described in Example 24d above] using the BL21(DE3) host (Novagen) rather than the BL21(DE3)pLysS host. The deletion of the pLysS plasmid in the BL21(DE3) host yielded higher levels of expression due to de-repression of the plasmid's T7-lac promoter. However, the yield of affinity-purified soluble recombinant protein was very low (approximately 600 µg/ liter culture) when purified under conditions identical to those described in Example 24d above. This result was due to the fact that expression in the BL21(DE3) host yielded very high level expression of the pHisBot protein as insoluble inclusion bodies as shown by SDS-PAGE analysis of protein prepared from induced BL21(DE3) cultures (Figure 29, lanes 1-7, described below). These results demonstrate that the pHisBot protein is not inherently toxic to *E. coli* cells and can be expressed to high levels using the appropriate promoter/host combination.

Figure 29 shows a Coomassie blue stained SDS-PAGE gel (12.5% acrylamide) onto which extracts prepared from BL21(DE3) cells containing the pHisBot plasmid were loaded. Each lane was loaded with 2.5 µl protein sample mixed with 2.5 µl of 2X SDS sample buffer. The samples were handled as described in Example 22b. The following samples were applied to the gel. Lanes 1-7 contain protein isolated from the BL21(DE3) host. Lanes 8-14 contain proteins isolated from the BL21(DE3)pLysS host. Total protein was loaded in lanes 1, 2, 4, 6, 8, 10 and 12. Soluble protein was loaded in Lanes 3, 5, 7, 9, 11 and 13. Lane 1 contains protein from uninduced host cells. Lanes 2-13 contain protein from host cells induced for 3 hours. IPTG was added to a final concentration of 0.1 mM (Lanes 6-7), 0.3 mM (Lanes 4-5) or 1.0 mM (Lanes 2, 3, 8-13). The cultures were grown in LB broth (Lanes 8-9), 2X YT broth (Lanes 10-11) or terrific broth (Lanes 1-7, 12-13). The pHisBot protein seen in Lanes 3, 5 and 7 is insoluble protein which spilled over from Lanes 2, 4 and 6, respectively. High molecular weight protein markers (BioRad) were loaded in Lane 14.

A variety of expression conditions were tested to determine if the BL21(DE3) host could be utilized to express soluble pHisBot protein at suitably high levels (i.e., about 10 mg/ml). The conditions altered were temperature (growth at 37 or 30°C), culture medium (2X YT, LB or Terrific broth) and inducer levels (0.1, 0.3 or 1.0 mM IPTG). All combinations of these variables were tested and the induction levels and solubility was then

assessed by SDS-PAGE analysis of total and soluble extracts [prepared from 1 ml samples as described in Williams et al., (1994), supra].

All cultures were grown in 15 ml tubes (Falcon #2057). All culture medium was prewarmed overnight at the appropriate temperature and were supplemented with 100 μg/ml ampicillin and 0.2% glucose. Terrific broth contains 12 g/l bacto-tryptone. 24 g/l bacto-yeast extract and 100 ml/l of a solution comprising 0.17 M KH₂PO₄, 0.72 M K₂HPO₄. Cultures were grown in a incubator on a rotating wheel (to ensure aeration) to an OD₆₀₀ of approximately 0.4, and induced by the addition of IPTG. In all cases, high level expression of insoluble pHisBot protein was observed, regardless of temperature, medium or inducer concentration.

5

10

15

20

25

30

The effect of varying the concentration of IPTG upon 2X YT cultures grown at 23°C was then investigated. IPTG was added to a final concentration of either 1 mM. 0.1 mM. 0.05 mM or 0.01 mM. At this temperature, similar levels of pHis Bot protein was induced in the presence of either 1 or 0.1 mM IPTG; these levels of expression was lower than that observed at higher temperatures. Induced protein levels were reduced at 0.05 mM IPTG and absent at 0.01 mM IPTG (relative to 1.0 and 0.1 mM IPTG inductions at 23°C). However, no conditions were observed in which the induced pHisBot protein was soluble in this host. Thus, although expression levels are superior in the BL21(DE3) host (as compared to the BL21(DE3)pLysS host), conditions that facilitate the production of soluble protein in this host could not be identified.

These results demonstrate that production of soluble pHisBot protein was achieved using the BL21(DE3)pLysS host in conjunction with the T7-lac promoter.

ii) Effect Of Varying Temperature, Medium And IPTG Concentration And Length Of Induction

The effect growing the host cells in various mediums upon the expression of recombinant botulinal protein from the pHisBot expression construct [in the BL21(DE3)pLysS host] was investigated. BL21(DE3)pLysS cells containing the pHisBot plasmid were grown in either LB, 2X YT or Terrific broth at 37°C. The cells were induced using 1 mM IPTG for a 3 hr induction period. Expression of pHisBot protein was found to be the highest when the cells were grown in 2X YT broth (see Figure 29, lanes 8-13).

The cells were then grown at 30°C in 2X YT broth and the concentration of IPTG was varied from 1.0, 0.3 or 0.1 mM and the length of induction was either 3 or 5 hours.

Expression of pHisBot protein was similar at all 3 inducer concentrations utilized and the levels of induced protein were higher after a 5 hr induction as compared to a 3 hr induction.

Using the conditions found to be optimal for the expression of pHisBot protein, a large scale culture was grown in order to provide sufficient material for a large scale purification of the pHisBot protein. Three 1 liter cultures were grown in 2X YT medium containing 100 µg/ml ampicillin, 34 µg/ml chloramphenicol and 0.2% glucose. The cultures were grown at 30°C and were induced with 1.0 mM IPTG for a 5 hr period. The cultures were harvested and a soluble lysate were prepared as described in Example 18. A large scale purification was performed as described in Example 24d with the exception that except the soluble lysate was batch absorbed for 3 hours rather than for 1 hour. The final yield was 13 mg pHisBot protein/liter culture. The pHisBot protein represented 0.75% of the total soluble protein.

The above results demonstrate growth conditions under which soluble pHisBot protein is produced (i.e., use of the BL21(DE3)pLysS host, 2X YT medium, 30°C, 1.0 mM IPTG for 5 hours).

15

5

10

b) Optimization Of Purification Parameters

For optimization of purification conditions, large scale cultures (3 X 1 liter) were grown at 30°C and induced with 1 mM IPTG for 5 hours as described above. The cultures were pooled, distributed to centrifuge bottles, cooled and pelleted as described in Example 24d. The cell pellets were frozen at -70°C until used. Each cell pellet represented 1/3 of a liter starting culture and individual bottles were utilized for each optimization experiment described below. This standardized the input bacteria used for each experiment, such that the yields of affinity purified pHisBot protein could be compared between different optimization experiments.

25

30

20

i) Binding Specificity (pH Protonation)

A lysate of pHisBot culture was prepared in PBS (pH 8.0) and applied to a 3 ml Ni-NTA column equilibrated in PBS (pH 8.0) using a flow rate of 0.2 ml/min (3-4 column volumes/hr) using an Econo chromatography system (BioRad). The column was washed with PBS (pH 8.0) until the absorbance (OD₂₈₀) of the clute was at baseline levels. The flow rate was then increased to 2 ml/min and the column was equilibrated in PBS (pH 7.0). A pH gradient (pH 7.0 to 4.0 in PBS) was applied in order to clute the bound pHisBot protein from the column. Fractions were collected and aliquots were resolved on SDS-PAGE gels. The

PAGE gels were subjected to Western blotting and the pHisBot protein was detected using a chicken anti-C. *botulinum* Type A toxoid antibody as described in Example 22.

From the Western blot analysis it was determined that the pHisBot protein begins to elute from the Ni-NTA column at pH 6.0. This is consistent with the predicted elution of a His-tagged protein monomer at pH 5.9.

These results demonstrate that the pH at which the pHisBot protein is protonated (released) from Ni-NTA resin in PBS buffer is pH 6.0.

ii) Binding Specificity (Imidazole Competition)

10

15

5

In order to define purification conditions under which the native *E. coli* proteins could be removed from the Ni-NTA column while leaving the pHisBot protein bound to the column, the following experiment was performed. A lysate of pHisBot culture was prepared in 50 mM NaHPO₄, 0.5 M NaCl, 8 mM imidazole (pH 7.0). This lysate was applied to a 3 ml Ni-NTA column equilibrated in 50 mM NaHPO₄, 0.5 M NaCl (pH 7.0) using an Econo chromatography system (BioRad). A flow rate of 0.2 ml/min (3-4 column volumes/hr) was utilized. The column was washed with 50 mM NaHPO₄, 0.5 M NaCl (pH 7.0) until the absorbance of the elute returned to baseline. The flow rate was then increased to 2 ml/min.

The column was eluted using an imidazole step gradient [in 50 mM NaHPO₄, 0.5 M NaCl (pH 7.0)]. Elution steps were 20 mM, 40 mM, 60 mM, 80 mM, 100 mM, 200 mM, 1.0 M imidazole, followed by a wash using 0.1 mM EDTA (to strip the nickel from the column and remove any remaining protein). In each step, the wash was continued until the OD₂₈₀ returned to baseline. Fractions were resolved on SDS-PAGE gels. Western blotted, and pHisBot protein detected using a chicken anti-*C. botulinum* Type A toxoid antibody as described in Example 22. Duplicate gels were stained with Coomassie blue to detect eluted protein in each fraction.

25

20

The results of the PAGE analysis showed that most of the non-specifically binding bacterial protein was removed by the 20 mM imidiazole wash, with the remaining bacterial proteins being removed in the 40 and 60 mM imidazole washes. The pHisBot protein began to elute at 100 mM imidazole and was quantitatively eluted in 200 mM imidazole.

30

These results precisely defined the window of imidazole wash stringency that optimally removes *E. coli* proteins from the column while specifically retaining the pHisBot protein in this buffer. These results provided conditions under which the pHisBot protein can be purified free of contaminating host proteins.

iii) Purificati n Buffers And Optimized Purification Pr tocols

A variety of purification parameters were tested during the development of an optimized protocol for batch purification of soluble pHisBot protein. The results of these analyses are summarized below.

Batch purifications were performed (as described in Example 24d) using several buffers to determine if alternative buffers could be utilized for binding of the pHisBot protein to the Ni-NTA column. It was determined that quantitative binding of pHisBot protein to the Ni-NTA resin was achieved in either Tris-HCl (pH 7.9) or NaHPO₄ (pH 8.0) buffers.

Binding of the pHisBot protein in NaHPO₄ buffer was not inhibited using 5 mM, 8 mM or 60 mM imidazole. Quantitative elution of bound pHisBot protein was obtained in buffers containing 50 mM NaHPO₄, 0.3 M NaCl (pH 3.5-4.0), with or without 10% glycerol. However, quantitation of soluble affinity purified pHisBot protein before and after a freeze thaw (following several weeks storage of the affinity purified elute at -20°C) revealed that 94% of the protein was recovered using the glycerol-containing buffer, but only 68% of the protein was recovered when the buffer lacking glycerol was employed. This demonstrates that glycerol enhanced the solubility of the pHisBot protein in this low pH buffer when the eluted protein was stored at freezing temperatures (e.g., -20°C). Neutralization of pH by addition of NaH₂PO₄ buffer did not result in obvious protein precipitation.

20

25

30

5

10

15

It was determined that quantitative binding of pHisBot protein using the batch format occurred after 3 hrs (Figure 30), but not after 1 hr of binding at 4°C (the resin was stirred during binding). Figure 30 depicts a Coomaisse blue stained SDS-PAGE gel (7.5% acrylamide) containing samples of proteins isolated during the purification of pHisBot protein from lysate prepared from the BL21(DE3)pLysS host. Each lane was loaded with 5 µl of protein sample mixed with 5 µl of 2X sample buffer and processed as described in Example 22b. Lane 1 contains high molecular weight protein markers (BioRad). Lanes 2 and 3 contain protein eluted from the Ni-NTA resin. Lane 4 contains soluble protein after a 3 hr batch incubation with the Ni-NTA resin. Lanes 5 and 6 contain soluble and total protein, respectively. Figure 30 demonstrates that the pHisBot protein is completely soluble [compare Lanes 5 and 6 which show that a similar amount of the 50 kD pHisBot protein is seen in both: if a substantial amount (greater than 20%) of the pHisBot protein were partially insoluble in the host cell, more pHisBot protein would be seen in lane 6 (total protein) as compared to lane 5 (soluble protein)]. Figure 30 also demonstrates that the pHisBot protein is

completely removed from the lysate after batch absorption with the Ni-NTA resin for 3 hours (compare Lanes 4 and 5).

The reported high affinity interaction of the Ni-NTA resin with His-tagged proteins (K_d= 1 x 10⁻¹³ at pH 8.0) suggested that it should be possible to manipulate the resin-protein complexes without significant release of the bound protein. Indeed, it was determined that after the recombinant protein was bound to the Ni-NTA resin, the resin-pHisBot protein complex was highly stable and remained bound following repeated rounds of centrifugation of the resin for 2 min at 1600 x g. When this centrifugation step was performed in a 50 ml tube (Falcon), a tight resin pellet formed. This allowed the removal of spent soluble lysate by pouring off the supernatant followed by resuspension of the pellet in wash buffer. Further washes can be performed by centrifugation. The ability to perform additional washes permits the development of protocols for batch absorption of large volumes of lysate with removal of the lysate being performed simply by centrifugation following binding of the recombinant protein to the resin.

. 5

10

15

20

25

30

A simplified, integrated purification protocol was developed as follows. A soluble lysate was made by resuspending the induced cell pellet in binding buffer [50 mM NaHPO4, 0.5 M NaCl, 60 mM imidazole (pH 8.0)], sonicating 4 x 20 sec and centrifuging for 20 min at 10.000 x g. NP-40 was added to 0.1% and Ni-NTA resin (equilibrated in binding buffer) was added. Eight milliliters of a 1:1 slurry (resin:binding buffer) was used per liter of starting culture. The mixture was stirred for 3 hrs at 4°C. The slurry was poured into a column having a 1 cm internal diameter (BioRad), washed with binding buffer containing 0.1% NP40, then binding buffer until baseline was established (these steps may alternatively be performed by centrifugation of the resin, resuspension in binding buffer containing NP40 followed by centrifugation and resuspension in binding buffer). Imidazole was removed by washing the resin with 50 mM NaHPO4, 0.3M NaCl (pH 7.0). Protein bound to the resin was eluted using the same buffer (50 mM NaHPO4, 0.3M NaCl) having a reduced pH (pH 3.5-4.0).

A pilot purification was performed following this protocol and yielded 18 mg/liter affinity-purified pHisBot. The pHisBot protein was greater than 90% pure as estimated by Coomassie staining of an SDS-PAGE gel. This represents the highest observed yield of soluble affinity-purified pHisBot protein and this protocol eliminates the need for separate imidazole-containing binding and wash buffers. In addition to providing a simplified and efficient protocol for the affinity purification of recombinant pHisBot protein, the above

results provide a variety of purification conditions under which pHisBot protein can be isolated.

EXAMPLE 26

5

The pHisBot Protein Is An Effective Immunogen

In Example 23 it was demonstrated that neutralizing antibodies are generated in mouse serum after nasal immunization with the pMBot protein. However, the pMBot protein was found to copurify with significant amounts of endotoxin which could not be easily removed. The pHisBot protein, in contrast, could be isolated free of significant endotoxin contamination making pHisBot a superior candidate for vaccine production. To further assess the suitability of pHisBot as a vaccine, the immunogenicity of the pHisBot protein was determined and a comparison of the relative immunogenicity of pMBot and pHisBot proteins in mice was performed as follows.

15

20

10

Two groups of eight BALBc mice were immunized with either pMBot protein or pHisBot protein using Gerbu GMDP adjuvant (CC Biotech). pMBot protein (in PBS containing 10 mM maltose) or pHisBot protein (in 50 mMNaHPO₄, 0.3 M NaCl, 10% glycerol, pH,4.0) was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received an IP injection of 100 µl antigen/adjuvant mix (50 µg antigen plus 1 µg adjuvant) on day 0. Mice were boosted as described above with the exception that the route of administration was IM on day 14 and 28. The mice were bled on day 77 and anti-C bouldinum Type A toxoid titers were determined using serum collected from individual mice in each group (as described in Example 23). The results are shown in Table 41.

5

10

15

25

30

35

TABLE 41

Anti-C bondman Type A Toxoid Serum tgG Titers In Individual Mice Immunized With pMBot or pHisBot Protein

	Preimmune ⁴					pl	MBot [:]	Bot pHisBot'				
Mouse #		Sample Dilution				Sample Dilution			Sample Dilation			
	1:50	1:250	1.1250	1:6250	1.50	1:250	1:1250	1:6250	1.50	1:250	1:1250	1.620
					0.678	0.190	0.055	0.007	1.574	(1,799	0.320	0 093
					1.161	0.931	0.254	0.075	1.513	0.829	0,409	0.134
					1.364	0.458	0.195	0.041	1 596	1.028	0.453	0.122
-1					1 622	1.189	0.334	0.067	1.552	0.840	0.348	0.090
:					1612	1.030	0.289	0.067	1 629	1.580	0.895	0.233
6					0.913	0.242	0.069	0.013	1.485	0.952	0 477	0.145
					0.910	0.235	0.058	0.014	1.524	0 725	0.269	0.069
8					0.747	0.234	0.058	0.014	1.274	0 427	0.116	0.029
Mean Titer	0.048	0 021	0.011	0.002	1 133	0.564	0 164	0.037	1.518	0.896	0.411	0 14

The preummune sample represents the average from 2 sets of duplicate wells containing serum from a individual mouse immunized with recombinant Staphylococcus enterotoxin B. (SEB) antigen. This antigen is immunologically unrelated to C handman toxin and provides a control serum.

20 Werage of duplicate wells

The results shown above in Table 41 demonstrate that both the pMBot and pHisBot proteins are immunogenic in mice as 100% of the mice (8/8) in each group seroconverted from non-immune to immune status. The results also show that the average titer of anti-C. botulinum Type A toxoid IgG is 2-3 fold higher after immunization with the pHisBot protein relative to immunization with the pMBot protein. This suggests that the pHisBot protein may be a superior immunogen to the pMBot protein.

EXAMPLE 27

Immunization With The Recombinant pHisBot Protein Generates Neutralizing Antibodies

The results shown in Example 26 demonstrated that both the pHisBot and pMBot proteins were capable of inducing high titers of anti-C. boulinum type A toxoid-reactive antibodies in immunized hosts. The ability of the immune sera from mice immunized with either the pHisBot or pMBot proteins to neutralize C. boulinum type A toxoid in vivo was determined using the mouse neutralization assay described in Example 23b.

The two groups of eight BALBc mice immunized with either pMBot protein or pHisBot protein in Example 26 were boosted again one week after the bleeding on day 77. The boost was performed by mixing pMBot protein (in PBS containing 10 mM maltose) or pHisBot protein (in 50 mM NaHPO₄, 0.3 M NaCl, 10% glycerol, pH 4.0) with Gerbu adjuvant as described in Example 26. Each mouse received an IP injection of 100 µl antigen/adjuvant mix (50 µg antigen plus 1 µg adjuvant). The mice were bled 6 days after this boost and the serum from mice within a group was pooled. Serum from preimmune mice was also collected (this serum is the same serum described in the footnote to Table 41).

5

10

15

20

25

30

The presence of neutralizing antibodies in the pooled or preimmune serum was detected by challenging mice with 5 LD₅₀ units of type A toxin mixed with 100 µl of pooled serum. The challenge was performed by mixing (per mouse to be injected) 100 µl of serum from each pool with 100 µl of purified type A toxin standard (50 LD₅₀ /ml prepared as described in Example 23b) and 500 µl of gel-phosphate. The mixtures were incubated for 30 min at room temperature with occasional mixing. Each of four mice were injected IP with the mixtures (0.7 ml/mouse). The mice were observed for signs of botulism for 72 hours. Mice receiving toxin mixed with serum from mice immunized with either the pHisBot or pMBot proteins showed no signs of botulism intoxication. In contrast, mice receiving preimmune serum died in less than 24 hours.

These results demonstrate that antibodies capable of neutralizing C. botulinum type A toxin are induced when either of the recombinant C. botulinum C fragment proteins pHisBot or pMBot are used as immunogens.

EXAMPLE 28

Cloning And Expression Of The C Fragment of C. botulinum Serotype A Toxin In E. coli Utilizing A Native Gene Fragment

In Example 22 above, a synthetic gene was used to express the C fragment of C. botulinum serotype Λ toxin in E. coli. The synthetic gene replaced non-preferred (i.e., rare) codons present in the C fragment gene with codons which are preferred by E. coli. The synthetic gene was generated because it was been reported that genes which have a high Λ/T content (such as most clostridial genes) creates expression difficulties in E. coli and yeast. Furthermore, LaPenotiere et al. suggested that problems encountered with the stability (non-fusion constructs) and solubility (MBP fusion constructs) of the C fragment of C. botulinum

serotype A toxin when expressed in E. coli was most likely due to the extreme A/T richness of the native C. hotulinum serotype A toxin gene sequences (LaPenotiere, et al., supra).

In this example, it was demonstrated that successful expression of the C fragment of C. hotulinum type A toxin gene in E. coli does not require the elimination of rare codons (i.e., there is no need to use a synthetic gene). This example involved a) the cloning of the native C fragment of the C. hotulinum serotype A toxin gene and construction of an expression vector and b) a comparison of the expression and purification yields of C hotulinum serotype A C fragments derived from native and synthetic expression vectors.

5

10

15

20

25

30

a) Cloning Of The Native C Fragment Of The C. botulinum
Serotype A Toxin Gene And Construction Of An Expression
Vector

The serotype A toxin gene was cloned from C. botulinum genomic DNA using PCR amplification. The following primer pair was employed: 5'-CGCCATGGCTAG ATTATTATCTACATTTAC-3' (5' primer, Neol site underlined: SEQ ID NO:29) and 5'-GCAAGCTTCTTGACAGACTCATGTAG-3' (3' primer, HindIII site underlined: SEQ ID NO:30). C. botulinum type A strain was obtained from the American Type Culture Collection (ATCC#19397) and grown under anaerobic conditions in Terrific broth medium. High molecular-weight C. botulinum DNA was isolated as described in Example 11. The integrity and yield of genomic DNA was assessed by comparison with a serial dilution of uncut lambda DNA after electrophoresis on an agarose gel.

The gene fragment was cloned by PCR utilizing a proofreading thermostable DNA polymerase (native *Pfu* polymerase). PCR amplification was performed using the above primer pair in a 50μl reaction containing 10mM Tris-HCl (pH 8.3). 50mM KCl. 1.5mM MgCl₂, 200μM each dNTP, 0.2μM each primer, and 50ng *C. hotulinum* genomic DNA. Reactions were overlaid with 100μl mineral oil, heated to 94°C 4 min. 0.5μl native *Pfu* polymerase (Stratagene) was added, and thirty cycles comprising 94°C for 1 min, 50°C for 2 min. 72°C for 2 min were carried out followed by 10 min at 72°C. An aliquot (10μl) of the reaction mixture was resolved on an agarose gel and the amplified native C fragment gene was gel purified using the Prep-A-Gene kit (BioRad) and ligated to pCRScript vector DNA (Stratagene). Recombinant clones were isolated and confirmed by restriction digestion, using standard recombinant molecular biology techniques [Sambrook *et al.* (1989), *supra*]. In addition, the sequence of approximately 300 bases located at the 5° end of the C fragment

coding region were obtained using standard DNA sequencing methods. The sequence obtained was identical to that of the published sequence.

5

10

15

20

25

30

An expression vector containing the native *C. hotulinum* serotype A C fragment gene was created by ligation of the *Ncol-Hin*dIII fragment containing the C fragment gene from the pCRScript clone to *Nhel-Hin*dIII restricted pETHisa vector (Example 18b). The *Ncol* and *Nhel* sites were filled in using the Klenow enzyme prior to ligation: these sites were thus blunt-end ligated together. The resulting construct was termed pHisBotA (native). pHisBotA (native) expresses the *C. hotulinum* serotype A C fragment with a his-tagged N terminal extension which has the following sequence:

MctGlyHisHisHisHisHisHisHisHisHisSerSerGlyHis*IleGluGlyArg*His<u>MetAla</u> (SEQ ID NO:24), where the underlining represents amino acids encoded by the *C. botulinum* C fragment gene (this N terminal extension contains the recognition site for FactorXa protease, shown in italics, which can be employed to removed the polyhistdine tract from the N-terminus of the fusion protein). The pHisBot (native) construct expresses the identical protein as the pHisBot construct (Ex. 24c; herein after the pHisBotA) which contains the synthetic gene.

The predicted DNA sequence encoding the native *C. hotulinum* serotype A *C* fragment gene contained within pHisBotA (native) is listed in SEQ ID NO:31 [the start of translation (ATG) is located at nucleotides 108-110 and the stop of translation (TAA) is located at nucleotides1494-1496 in SEQ ID NO:31] and the corresponding amino acid sequence is listed in SEQ ID NO:26 (*i.e.*, the same amino acid sequence as that produced by pHisBotA containing synthetic gene sequences).

b) Comparison Of The Expression And Purification Yields Of C. botulinum Serotype A C Fragments Derived From Native And Synthetic Expression Vectors

Recombinant plasmids containing either the native or the synthetic *C. hotulinum* serotype A C fragment genes were transformed into *E. coli* strain Bl21(DE3) pLysS and protein expression was induced in 1 liter shaker flask cultures. Total protein extracts were isolated, resolved on SDS-PAGE gels and *C. hotulinum* C fragment protein was identified by Western analysis utilizing a chicken anti-*C. hotulinum* serotype A toxoid antiserum as described in Example 22.

Briefly. 1 liter (2XYT + 100 μg/ml ampicillin and 34 μg/ml chloramphenicol) cultures of bacteria harboring either the pHisBotA (synthetic) or pHisBotA (native) plasmids in the Bl21(DE3) pLysS strain were induced to express recombinant protein by addition of IPTG to 1mM. Cultures were grown at 30-32°C. IPTG was added when the cell density reached an OD₆₀₀ 0.5-1.0 and the induced protein was allowed to accumulate for 3-4 hrs after induction.

. 5

10

15

20

25

30

The cells were cooled for 15 min in a ice water bath and then centrifuged for 10 min at 5000 rpm in a JA10 rotor (Beckman) at 4°C. The cell pellets were resuspended in a total volume of 40 mls 1X binding buffer (40 mM imidazole, 0.5 M NaCl, 50 mM NaPO₄, pH 8.0), transferred to two 50 ml Oakridge tubes and frozen at -70°C for at least 1 hr. The tubes were then thawed and the cells were lysed by sonication (using four successive 20 second bursts) on ice. The suspension was clarified by centrifugation 20-30 min at 9,000 rpm (10,000g) in a JA-17 rotor. The soluble lysate was batch absorbed to 7 ml of a 1:1 slurry of NiNTA resin:binding buffer by stirring 2-4 hr at 4°C. The slurry was centrifuged for 1 min at 500g in 50 ml tube (Falcon), resuspended in 5 mls binding buffer and poured into a 2.5 cm diameter column (BioRad). The column was attached to a UV monitor (ISCO) and the column was washed with binding buffer until a baseline was established. Imidazole was removed by washing with 50mM NaPO₄, 0.3 M NaCl, 10% glycerol, pH 7.0 and bound protein was eluted using 50mM NaPO₄, 0.3 M NaCl, 10% glycerol, pH 3.5-4.0.

The eluted proteins were stored at 4°C. Samples of total, soluble, and eluted proteins were resolved by SDS-PAGE. Protein samples were prepared for electrophoresis by mixing 1µl total (T) or soluble (S) protein with 4 µl PBS and 5 µl 2X SDS-PAGE sample buffer, or 5 µl eluted (E) protein and 5 µl 2X SDS-PAGE sample buffer. The samples were heated to 95°C for 5 min, then cooled and 5 or 10 µls were loaded on 12.5% SDS-PAGE gels. Broad range molecular weight protein markers (BioRad) were also loaded to allow the MW of the identified fusion proteins to be estimated. After electrophoresis, protein was detected either generally by staining gels with Coomassie blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein.

For Western blot analysis, the gels were blotted, and protein transfer was confirmed by Ponceau S staining as described in Example 22. After blocking the blots for 1 hr at room temperature in blocking buffer (PBST and 5% milk), 10 ml of a 1/500 dilution of an anti-C. botulinum toxin A IgY PEG prep (Ex. 3) in blocking buffer was added and the blots were incubated for an additional hour at room temperature. The blots were washed and developed using a rabbit anti-chicken alkaline phosphatase conjugate (Boehringer Mannheim) as the

secondary antibody as described in Ex. 22. This analysis detected *C. hotulinum* toxin A-reactive proteins in the pHisBotA (native and synthetic) protein samples (corresponding to the predicted full length proteins identified by Coomassie staining).

A gel containing proteins expressed from the pHisBot and pHisBot (native) constructs during various stages of purification and stained with Coomassie blue is shown in Figure 31. In Figure 31, lanes 1-4 and 9 contain proteins expressed by the pHisBotA construct (i.e., the synthetic gene) and lanes 5-8 contain proteins expressed by the pHisBotA (native) construct. Lanes 1 and 5 contain total protein extracts; lanes 2 and 6 contain soluble protein extracts; lanes 3 and 7 contain proteins which flowed through the NiNTA columns; lanes 4, 8 and 9 contain protein eluted from the NiNTA columns and lane 10 contains molecular weight markers.

The above purification resulted in a yield of 3 mg (native gene) or 11 mg (synthetic gene) of affinity purified protein from a 1 liter starting culture, of which at least 90-95% of the protein was a single band of the predicted MW (50kd) and immunoreactivity for recombinant *C. botulinum* scrotype A C fragment protein. Other than the level of expression, no difference was observed between the native and the synthetic gene expression systems.

These results demonstrate that soluble C, botulinum serotype Λ C fragment protein can be expressed in E, coli and purified utilizing either native or synthetic gene sequences.

20

25

30

5

10

15

EXAMPLE 29

Generation Of Neutralizing Antibodies Using A Recombinant

C. botulinum Serotype A C Fragment Protein Containing A Six Residue His-Tag

In Example 27. neutralizing antibodies were generated utilizing the pHisBotA protein, which contains a histidine-tagged N-terminal extension comprising 10 histidine residues. To determine if the generation of neutralizing antibodies is dependent on the presence of this particular his-tag, a protein containing a shorter N-terminal extension (comprising 6 histidine residues) was produced and tested for the ability to generate neutralizing antibodies. This example involved a) the cloning and expression of the p6HisBotA(syn) protein and b) the generation and characterization of hyperimmune serum.

a) Cloning And Expressi n Of The p6HisBotA(syn) Pr tein

• 5

10

15

20

25

30

The p6HisBotA(syn) construct was generated as described below: the term "syn" designates the presence of synthetic gene sequences. This construct expresses the C frgament of the C. hotulinum scrotype A toxin with a histidine-tagged N terminal extension having the following sequence: MetHisHisHisHisHisHisHisMetAla (SEQ ID NO:32): the amino acids encoded by the botulinal C fragment gene are underlined and the vector encoded amino acids are presented in plain type.

6XHis oligonucleotides [5'-TATGCATCACCATCACCATCA-3' (SEQ ID NO:33) and 5'-CATGTGATGGTGATGGTGATGCA-3' (SEQ ID NO:34) were annealed as follows. One microgram of each oligonucleotide was mixed in total of 20 μl 1X reaction buffer 2 (NEB) and the mixture was heated at 70°C for 5 min and then incubated at 42°C for 5 min. The annealed oligonucleotides were then ligated with gel purified *Ndel/HindIII* cleaved pET23b (T7 promoter) or pET21b (T7lac promoter) DNA and the gel purified *Ncol/HindIII* (C. hondinum serotype A C fragment synthetic gene fragment derived from pAlterBot (Ex. 22). Recombinant clones were isolated and confirmed by restriction digestion. The DNA sequence encoding the 6X his-tagged BotA protein contained within p6HisBotA(syn) is listed in SEQ ID NO:35. The amino acid sequence of the p6XHisBotA protein is listed in SEQ ID NO:36.

The resulting recombinant p6XHisBotA plasmid was transformed into the B1.21(DE3) pLysS strain, and 1 liter cultures were grown, induced and harvested as described in Example 28. His-tagged protein was purified as described in Example 28, with the following modifications. The binding buffer (BB) contained 5 mM imidazole rather than 40 mM imidazole and NP40 was added to the soluble lysate to a final concentration of 0.1%. The bound material was washed on the column with BB until the baseline was established, then the column was washed successively with BB+20 mM imidazole and BB+40 mM imidazole. The column was eluted as described in Example 28.

In the case of the pET23-derived expression system, high level expression of insoluble 6HisBotA protein was induced. The pET21-derived vector expressed lower levels of soluble protein that bound the NiNTA resin and eluted in the 40 mM imidazole wash rather than during the low pH elution. These results (i.e., low level expression of a soluble protein) are consistent with the results obtained with pHisBotA protein (Ex. 25); the pHisBotA construct, like the pET21-derived vector, contains the T7lac rather than T7 promoter.

The 6HisBotA protein thus clutes under less stringent conditions than the 10X histidine-containing pHisBot protein (100-200 mM imidazole: Ex. 25) presumably due to the

reduction in the length of the his-tag. The eluted protein was of the predicted size [i.e., slightly reduced in comparison to pHisBotA protein].

b) Generation And Characterization Of Hyperimmune Serum

5

10

15

20

25

Eight BALBc mice were immunized with purified 6HisBotA protein using Gerbu GMDP adjuvant (CC Biotech). The 40 mM imidazole elution was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received a subcutaneous injection of 100 μl antigen/adjuvant mix (12 μg antigen + 1 μg adjuvant) on day 0. Mice were subcutaneously boosted as above on day 14 and bled on day 28. Control mice received pHisBotB protein (prepared as described in Ex. 35 below) in Gerbu adjuvant.

Anti-C. botulinum serotype A toxoid titers were determined in serum from individual mice from each group using the ELISA described in Example 23a with the exception that the initial testing serum dilution was 1:100 in blocking buffer containing 0.5% Tween 20, followed by serial 5-fold dilutions into this buffer. The results of the ELISA demonstrated that seroconversion (relative to control mice) occurred in all 8 mice.

The ability of the anti-C. botulinum serotype A C fragment antibodies present in serum from the immunized mice to neutralize native C. botulinum type A toxin was tested using the mouse neutralization assay described in Example 23b. The amount of neutralizing antibodies present in the serum of the immunized mice was determined using serum antibody titrations. The various serum dilutions (0.01 ml) were mixed with 5 LD_{s0} units of C. botulinum type A toxin and the mixtures were injected IP into mice. The neutralizations were performed in duplicate. The mice were then observed for signs of botulism for 4 days. Undiluted serum was found to protect 100% of the injected mice while the 1:10 diluted serum did not. This corresponds to a neutralization titer of 0.05-0.5 IU/ml.

These results demonstrate that neutralizing antibodies were induced when the 6HisBotA protein was utilized as the immunogen. Furthermore, these results demonstrate that seroconversion and the generation of neutralizing antibodies does not depend on the specific N terminal extension present on the recombinant C. botulinum type A C fragment proteins.

EXAMPLE 30

Construction Of Vectors For The Expression Of His-Tagged

C. hotulinum Type A Toxin C Fragment Protein Using the Synthetic Gene

A number of expression vectors were constructed which contained the synthetic C. botulinum type A toxin C fragment gene. These constructs vary as to the promoter (T7 or T7lac) and repressor elements (laclq) present on the plasmid. The T7 promoter is a stronger promoter than is the T7lac promoter. The various constructs provide varying expression levels and varying levels of plasmid stability. This example involved a) the construction of expression vectors containing the synthetic C. botulinum type A C fragment gene and b) the determination of the expression level achieved using plasmids containing either the kanamycin resistance or the ampicillin resistance genes in small scale cultures.

a) Construction Of Expression Vectors Containing The Synthetic C. botulinum Type A C Fragment Gene

5

10

15

20

25

30

Expression vectors containing the synthetic C. botulinum type A C fragment gene were engineered to utilize the kanamycin resistance rather than the ampicillin resistance gene. This was done for several reasons including concerns regarding the presence of residual ampicillin in recombinant protein derived from plasmids containing the ampicillin resistance gene. In addition, ampicillin resistant plasmids are more difficult to maintain in culture: the β -lactamase secreted by cells containing ampicillin resistant plasmids rapidly degrades extracellular ampicillin, allowing the growth of plasmid-negative cells.

A second altered feature of the expression vectors is the inclusion of laclq gene in the plasmid. This repressor lowers expression from lac regulated promoters (the chromosomally located, lactose regulated T7 polymerase gene and the plasmid located T7lac promoter). This down regulates uninduced protein expression and can enhance the stability of recombinant cell lines. The final alteration to the vectors is the inclusion of either the T7 or T7lac promoters that drive high or moderate level expression of recombinant protein, respectively.

The expression plasmids were constructed as follows. In all cases, the protein expressed is the pHisBotA(syn) protein previously described, and the only differences between constructs is the alteration of the various regulatory elements described above.

i) Construction Of pHisB tA(syn) kan T7lac

5

10

15

20

25

The pHisBotA(syn) kan T7lac construct was made by inserting the Sapl/Xhol fragment containing the C. botulinum type A C fragment from pHisBotA(syn) into pET24 digested with Sapl/Xhol (Novagen: fragment contains kan gene and origin of replication). The desired construct was selected for kanamycin resistance and confirmed by restriction digestion.

ii) Construction Of pHisBotA(syn) kan laclq T7lac

The pHisBotA(syn) kan laclq T7lac construct was made by inserting the $\lambda bal/HindIII$ fragment containing the C. botulinum type A C fragment from pHisBotA(syn)kanT7lac into the pET24a vector digested with Xbal/HindIII. The resulting construct was confirmed by restriction digestion.

iii) Construction Of pHisBotA(syn) kan laclq T7

The pHisBotA(syn) kan laclq T7 construct was made by inserting the Xbal/HindIII fragment containing the C. botulinum type A C fragment from pHisBotA(syn) kan laclq T7lac into Xbal/HindIII-digested pHisBotB(syn) kan laclq T7 (described in Ex 37c below). The resulting construct was confirmed by restriction digestion.

b) Determination Of The Expression Level Achieved Using Plasmids Containing Either The Kanamycin Resistance Or The Ampicillin Resistance Genes In Small Scale Cultures

One liter cultures of pHisBotA(syn) kan T7lac/Bl21(DE3)pLysS and pHisBotA(syn) amp T7lac/Bl21(DE3)pLysS [this is the previously designated pHisBotA(syn) construct] were grown, induced and his-tagged proteins were purified as described in Example 28. No differences in yield or protein integrity/purity were observed.

These results demonstrate that the antigen induction levels from expression constructs were not affected by the choice of ampicillin versus kanamycin antibiotic resistance genes.

EXAMPLE 31

Fermentation Of Cells Expressing Recombinant Botulinal Proteins

a) Fermentation Culture Of Cells Expressing Recombinant Botulinal Proteins

. 5

10

15

20

25

30

Fermentation cultures were grown under the following conditions which were optimized for growth of the BL21(DE3) strains containing pET derived expression vectors. An overnight 1 liter feeder culture was prepared by inoculating of 1 liter media (in a 2L shaker flask) with a fresh colony grown on an LB kan plate. The feeder culture contained: 600 mls nitrogen source [20 gm yeast extract (BBL) and 40 gm tryptone (BBL)/600 mls]. 200 mls 5X fermentation salts (per liter: 48.5 gm K₂HPO₄, 12 gm NaH₂PO₄•H₂O, 5 gm NH₄Cl, 2.5 gm NaCl). 180 mls dH₂O, 20 mls 20% glucose, 2 mls 1 M MgSO₄, 5 mls 0.05M CaCl, and 4 mls of a 10 mg/ml kanamycin stock. All solutions were sterilized by autoclaving, except the kanamycin stock which was filter sterilized.

An aliquot (5 ml) of the feeder culture broth was removed prior to inoculation, and grown for 2 days at 37°C as a culture broth sterility control. Growth was not observed in this control culture in any of the fermentations performed.

The inoculated feeder culture was grown for 12-15 hrs (ON) at 30-37°C. Care was taken to prevent oversaturation of this culture. The saturated feeder culture was added to 10L of fermentation media in fermenter (BiofloIV, New Brunswick Scientific, Edison, NJ) as follows. The fermenter was sterilized 120 min at 121°C with dH₂O. The sterile water was removed, and fermentation media added as follows: 6 liters nitrogen source, 2 liters 5X fermentation salts, 2 liters 2% glucose, 20 mls 1 M MgSO₄, 50 mls 0.05 M CaCl₂, 2.5-3.5 mls Macol P 400 antifoam (PPG Industries Inc., Gurnee, IL), 40 mls 10mg/ml kanamycin and 10 mls trace elements (8 gm FeSO₄•7H₂O, 2 gm MnSO₄•H₂O, 2 gm AlCl₃•6H₂O, 0.8 gm CoCl•6H₂O, 0.4 gm ZnSO₄•7H₂O, 0.4 gm Na₂MoO₄•2H₂O, 0.2 gm CuCl₂•2H₃O, 0.2 gm NiCl₃, 0.1 gm H₃BO₄/200mls 5 M HCl). All solutions were sterilized by autoclaving, except the kanamycin stock which was filter sterilized. Fermentation media was prewarmed to 37°C before the addition of the feeder culture.

After the addition of the feeder culture, the culture was fermented at 37°C, 400 rpm agitation, and 10 l/min air sparging. The DO₂ control was set to 20% PID and dissolved oxygen levels were controlled by increasing the rate of agitation from 400-850 rpm under DO₂ control. DO₂ levels were maintained at greater than or equal to 20% throughout the

5

10

15

20

25

30

entire fermentation. When agitation levels reached 500-600 rpm the temperature was lowered to 30°C to reduce the oxygen consumption rate. Culture growth was continued until endogenous carbon sources were depleted. In these fermentations, glucose was depleted first [monitored with a glucose monitoring kit (Sigma)], followed by assimilation of acetate and other acidic carbons [monitored using an acetate test kit (Boehringer Mannheim)]. During the assimilation phase, the pH rose from 6.6-6.8 (starting pH) to 7.4-7.5, at which time the bulk of the remaining carbon source was depleted. This was signaled by a drop in agitation rate (from a maximum of 700-800 rpm) and a rise in DO₂ levels >30%. This corresponds to a OD en reading of 18-20/ml. At this point a fed batch mode was initiated, in which a feed solution of 50% glucose was added at a rate of approximately 4 gm glucose/liter/hr. The pH was adjusted to 7.0 by the addition of 25% H₃PO₄ (approximately 60 mls). Culture growth was continued and reached peak oxygen consumption within the next 3 hrs of growth (while the remaining residual non-glucose carbon sources were assimilated). This phase is characterized by a slow increase in pH, and air sparging was increased to 15L/min, to keep the maximum rpm below 850. Once the residual acidic carbon sources are depleted the agitation rate decreases to 650-750 rpm and the pH begins to drop. pH control was maintained at 7.0 PID by regulated pump addition of a sterile 4M NaOH solution which was consumed at a steady rate for the remainder of the fermentation. Growth was continued at 30°C, and the cultures were grown linearly at a growth rate of 4-7 OD, units/hr. to at least 81.5 OD₆₀₀ units/ml (>30g/l dry cell weight) without induction. Antifoam (a 1:1 dilution with filter sterilized 100% ethanol) was added as necessary throughout the fermentation to prevent foaming.

During the fed batch mode, glucose was assimilated immediately (concentration in media consistently less than 0.1 gm/liter) and acetate was not produced in significant levels by the pET plasmid/BL21(DE3) cell lines tested (approximately 1 gm/liter at end of fermentation: this is lower than that observed in harvests from shaker flask cultures utilizing the same strains). This was fortuitous, since high levels of acetate has been shown to inhibit induction levels in a variety of expression systems. The above described conditions were found to be highly reproducible between fermentations and utilizing different expression plasmids. As a result, glucose and acetate level monitoring were no longer preformed during fermentation.

b) Induction Of Fermentation Cultures

Induction with IPTG (250 mg-10 gms, depending on the expression vector and experiment) was initiated 1-3 hrs after initiation of the glucose feed (30-50 OD_{600}/ml). The growth rate after induction was monitored on a hourly basis. Aliquots (5-10 ml) of cells were harvested at the time of induction, and at hourly intervals post-induction. Optical density readings were determined by measuring the absorbance at 600 nm of 10 μ l culture in 990 μ l PBS versus a PBS control. The growth rate after induction was found to vary depending on the expression system utilized.

c) Monitoring Of Fermentation Cultures

Fermentation cultures were monitored using the following control assays.

i) Colony Forming Ability

An aliquots of cells were removed from the cultures at each timepoint sampled (uninduced and at various times after induction) were serially diluted in PBS (dilution 1=15 μ l cells/3 ml PBS, dilution 2=15 μ l of dilution 1/3 ml PBS, dilution 3=3 or 6 μ l of dilution 2/3mls PBS) and 100 μ l of dilution 3 was plated on an LB or TSA (trypticase soy agar) plate. The plates were incubated ON at 37°C and then the colonies are counted and scored for macro or micro growth.

20

25

30

. 5

10

15

ii) Phenotypic Characterization

Colonies growing on LB or TSA plates (above) from uninduced and induced timepoints were replica plated onto LB+kan, LB+chloramphenicol (for fermentations utilizing LysS or pACYCGro plasmids). LB+kan+1mM IPTG and LB plates, in this order. The plates were grown 6-8 hrs at 37°C and growth was scored on each plate for a minimum of 40-50 well isolated colonies. The percentage of cells retaining the plasmid at time of induction (i.e., uninduced cultures immediately prior to the addition of IPTG) was determined to be the # colonies LB+Kan (or chloramphenicol) plate/# colonies LB plate X 100%. The percentage of cells with mutated pET plasmids was determined to be the # colonies LB+Kan+IPTG plate/# colonies LB plate X 100%. Colonies on all LB plates were scored morphologically for E. coli phenotype as a contamination control. Morphologically detectable contaminant colonies were not detected in any fermentation.

iii) Recombinant B tA Protein Induction

A total of 10 OD₆₀₀ units of cells (e.g., 200 µl of cells at OD₆₀₀=50/ml) were removed from each timepoint sample to a 1.5 ml microfuge tube and pelleted for 2 min at maximum rpm in a microfuge. The pellets were resuspended in 1 ml of 50 mM NaHPO₄, 0.5 M NaCl, 40mM imidazole buffer (pH 6.8) containing 1 mg/ml lysozyme. The samples were incubated for 20 min at room temperature and stored ON at -70°C. Samples were thawed completely at room temperature and sonicated 2 X 10 seconds with a Branson Sonifier 450 microtip probe at # 3 power setting. The samples were centrifuged for 5 min at maximum rpm in a microfuge.

10

15

5

An aliquot (20 µl) of the protein samples were removed to 20 µl 2X sample buffer, before or after centrifugation, for total and soluble protein extracts, respectively. The samples were heated to 95°C for 5 min, then cooled and 5 or 10 µl were loaded onto 12.5% SDS-PAGE gels. High molecular weight protein markers (BioRad) were also loaded to allow for estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining gels with Coomassie blue, or specifically, by blotting onto nitrocellulose (as described in Ex. 28) for Western blot detection of specific his-tagged proteins utilizing a NiNTA-alkaline phosphatase conjugate exactly as described by the manufacturer (Qiagen).

20

25

30

iv) Recombinant Antigen Purification

At the end of each fermentation run, 1-10 liters of culture were harvested from the fermenter and the bacterial cells were pelleted by centrifugation at 6000 rpm for 10 min in a JA10 rotor (Beckman). The cell pellets were stored frozen at -70°C or utilized immediately without freezing. Cell pellets were resuspended to 15-20% weight to volume in resuspension buffer (generally 50 mM NaPO₄, 0.5 M NaCl, 40mM imidazole, pH 6.8) and lysed utilizing either sonication or high pressure homogenization.

For sonication, the resuspension buffer was supplemented with lysozyme to 1 mg/ml, and the suspension was incubated for 20 min, at room temp. The sample was then frozen ON at -70°C, thawed and sonicated 4 X 20 seconds at microtip maximum to reduce viscosity. For homogenization, the cells were lyzed by 2 passes through a homogenizer (Rannie Mini-lab type 8.30 H) at 600 Bar. Cell lysates were clarified by centrifugation for 30 min at 10,000 rpm in a JA10 rotor.

For IDA chromatography, samples were flocculated utilizing polyethyleneimine (PEI) prior to centrifugation. Cell pellets were resuspended in cell resuspension buffer (CRB: 50 mM NaPO₄, 0.5 M NaCl. 40 mM imidazole, pH 6.8) to create a 20% cell suspension (wet weight of cells/volume of CRB) and cell lysates were prepared as described above (sonication or homogenization). PEI (a 2% solution in dH₂O, pH 7.5 with HCl) was added to the cell lysate a final concentration of 0.2%, and stirred for 20 min at room temperature prior to centrifugation (8.500 rpm in JA10 rotor for 30 minutes at 4°C). This treatment removed RNA. DNA and cell wall components, resulting in a clarified, low viscosity lysate ("PEI clarified lysate").

- 5 -

10

15

20

25

30

His-tagged proteins were purified from soluble lysates by metal-chelate affinity chromatography using either a NiNTA resin (as described in Ex. 28) or an IDA (iminodiacetic acid) resin as described below.

thromatography system (ISCO). A 7 ml (small scale) or 70 ml (large scale) Chelating Sepharose Fast Flow (Pharmacia) affinity column was poured: in addition, a second guard column was poured and attached in line with the first column (to capture Ni ions that leached off the affinity column). The columns were washed with 3 column volumes of dH₂O. The guard column was then removed and the affinity column was washed with 0.3 M NiSO₄ until resistivity was established, then with dH₂O until the resistivity returned to baseline. The columns were reconnected and equilibrated with cell resuspension buffer (CRB; 50 mM NaPO₄, 0.5 M NaCl, 40 mM imidazole, pH 6.8). The clarified sample (in CRB) was loaded. Flow rates were 5 ml/min for small scale columns and 20 ml/min for large scale columns. After sample loading, the column was washed with CRB until a baseline established and bound protein was cluted with clution buffer (50 mM NaPO₄, 0.5 M NaCl, 800 mM imidazole, 20% glycerol, pH 6.8 or 8.0). Protein samples were stored at 4°C or -20°C. The yield of cluted protein was established by measuring the OD₂₈₀ of the clutions, with a 1 mg/ml solution of protein assumed to yield an absorbance reading of 2.0.

The IDA columns may be regenerated and reused multiple times (>10). To regenerate the column, the column was washed with 2-3 column volumes of H₂O, then 0.05 M EDTA until all of the blue/green color was removed followed by a wash with dH₂O. The IDA columns were sterilized with 0.1 M NaOH (using at least 3 column volumes but not more than 50 minutes contact time with column packing material), then washed with 3 column volumes 0.05 M NaPO₄, pH 5.0, then dH₂O and stored at room temperature in 20 % ethanol.

EXAMPLE 32

Construction Of A Folding Chaperone Overexpression System

Co-overexpression of the *E. coli* GroEL/GroES folding chaperones in a cell expressing a recombinant foreign protein has been reported to enhance the solubility of some foreign proteins that are otherwise insoluble when expressed in *E. coli* [Gragerouu *et al.* (1992) Proc. Natl. Acad. Sci. USA 89:10344]. The improvement in solubility is thought to be due to chaperone-mediated binding and unfolding of insoluble denatured proteins, thus allowing multiple attempts for productive refolding of recombinant proteins. By overexpressing the chaperones, the unfolding/refolding reaction is driven by excess chaperone, resulting, in some cases, in higher yields of soluble protein.

5

10

15

20

25

30

In this example, a chaperone overexpression system, compatible with pET vector expression systems, was constructed to facilitate testing chaperone-mediated solubilization of *C. hotulinum* type A proteins. This example involved the cloning of the GroEL/ES operon and construction of a pLysS-based chaperone hyperexpression system.

The GroEL/GroES operon was PCR amplified and cloned into the pCRScript vector as described in Example 28. The following primer pair was used: 5'-CGCAT ATGAATATTCGTCCATTGCATG-3' (SEQ ID NO:37) [5' primer, start codon of groES gene converted to Ndel site (underlined)] and 5'-GGAAGCTTGCAGGGCAAT TACATCATG (SEQ ID NO:38) (3' primer, stop codon of groEL gene italicized, engineered HindIII site underlined). Following amplification, the chaperone operon was excised as an Ndel/HindIII fragment and cloned into pET23b digested with Ndel and HindIII. This construction places the Gro operon under the control of the T7 promoter of the pET23 vector. The desired construct was confirmed by restriction digestion.

The T7 promoter-Gro operon-T7 terminator expression cassette was then excised as a BglII/BspEI (filled) fragment and cloned into BamHI (compatible with BglII)/HindIII (filled) cleaved pLysS plasmid (this removed the T7 lysozyme gene). The resulting construct was designated pACYCGro, since the plasmid utilizing the pACYC184 origin from the plysS plasmid. Proper construction was confirmed by restriction digestion.

pACYCGro was transformed into BL21(DE3), cultures were grown and induced with 1 mM IPTG as described in preceding examples. Total and soluble protein extracts were generated from cells removed before and after IPTG induction and were resolved on a 12.5 % SDS-PAGE gel and stained with Coomassie blue. This analysis revealed that high levels of

soluble GroEl and GroES proteins were made in the induced cells. These results demonstrated that the chaperone hyper-expression system was functional.

. 5

10

15

20

25

30

EXAMPLE 33

Growth Of BotA/pACYCGro Cell Lines In Fermentation Cultures

Induction of BL21(DE3) cells lacking the LysS plasmid which contained BotA expression constructs grown in shaker flask or fermentation culture resulted in the expression of primarily insoluble BotA protein. Fermentation cultures were performed to determine if the simultaneous overexpression of the Gro operon and recombinant *C. botulinum* type A proteins (BotA proteins) resulted in enhanced solubility of the recombinant BotA protein. This example involved the fermentation of pHisBotA(syn)kan laclq T7lac/pACYCGro BL21(DE3) and pHisBotA(syn)kan laclq T7/pACYCGro BL21(DE3) cell lines. The fermentations were repeated exactly as described in Example 31. Chloramphenicol (34 μg/ml) was included in the feeder and fermentation cultures.

a) Fermentation Of pHisBotA(syn)kan laclq <u>T7lac/pACYCGro</u> BL21(DE3) Cells

For fermentation of cells containing plasmids comprising the T7lac promoter, induction was with 2 gms IPTG at 1 hr post initiation of glucose feed. The OD₆₀₀ was 35 at time of induction, then 48.5, 61.5, 67 at 1-3 hrs post induction. Viable colony counts decreased from 0-3 hr induction [21 (13), 0, 0, 0; dilution 3 utilized 3 µl of dilution 2 cells] with numbers in parenthesis for the indicating microcolonies. Of 28 colonies scored at the time of induction, 23 retained the pHisBotA(syn)kan lacIq T7lac plasmid (kan resistant), 22 contained the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected). These results were indicative of very strong promoter induction, since colony viability dropped immediately after induction.

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and stained with Coomassie. High level induction of Gro chaperones was observed, but very low level expression of soluble BotA protein was observed, increasing from 1 to 4.0 hrs post induction (no expression detected in uninduced cells). The dramatically lower expression of the BotA antigen in the presence of chaperone may be due to promoter occlusion (i.e., the stronger T7 promoter on the chaperone plasmid is preferentially utilized).

b) Fermentation Of pHisBotA(syn)kan laclq <u>T7</u>/ pACYCGro BL21(DE3) Cells

5 .

10

15

20

25

30

A fermentation utilizing the T7-driven BotA expression plasmid was performed. Induction was with 1 gm IPTG at 2 hrs post initiation of glucose feed. The OD_{κm} was 41 at time of induction, then 51.5, 61.5, 61.5 and 66 at 1-4 hrs post induction. Viable colony counts decreased from 0-4 hrs induction [71, 1 (34), 1 (1), 1, 0; dilution 3 utilized 6 μl dilution 2 cells) with numbers in parenthesis for the uninduced timepoint indicating microcolonies. Of 65 colonies scored at the time of induction, all 65 retained both the pHisBotA(syn)kan lacIq T7 plasmid (kan resistant) and the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and stained with Coomassie. High level induction of Gro chaperones and moderate level expression of soluble BotA protein was observed, increasing from 1 to 4.0 hrs post induction (no expression detected in uninduced cells).

A PEI-clarified lysate (0.2% final cocnentration PEI) [850 ml from 130 gm cell pellet (2 liters fermentation harvest)] was purified on a large scale IDA column. A total of 78 mg of protein was eluted. Extracts from the purification were resolved on a 12.5% SDS-PAGE gel and stained with Coomassie. The elution was found to contain an approximately 1:1 mix of BotA/chaperone protein (Figure 32). PEI lysates prepared in this manner were typically 16 OD₂₈₀/ml. This was estimated to be 8 mg protein/ml of lysate (by BCA assay). Thus, the eluted recombinant BotA protein represented 0.55% of the total soluble cellular protein applied to the column.

In Figure 32, lane 1 contains molecular weight markers, lanes 2-9 contain extracts from pHisBotA(syn)kan laclq T7/pACYCGro/BL21(DE3) cells before or during purification on the IDA column. Lane 2 contains total protein extract; lane 3 contains soluble protein extract; lanes 4 and 5 contain PEI-clarified lysates (duplicates); lanes 6 and 7 contain flow-through from the IDA column (duplicates) and lanes 8 and 9 contain IDA column elute (lane 9 contains 1/10 the amount applied to lane 8).

These results demonstrate, that although the majority of the BotA protein produced was insoluble, 20 mg/liter of soluble recombinant BotA protein can be purified utilizing the pHisBotA(syn)kan laclq T7/pACYCGro/BL21(DE3) expression system.

EXAMPLE 34

Purification Of Recombinant BotA Protein From Folding Chaperones

In this example of size exclusion chromatography was used to purify the recombinant BotA protein away from the folding chaperones and imidazole present in the IDA-purified material (Ex. 33).

5

10

15

20

25

30

To enhance the solubility of the recombinant BotA protein during scale-up, the protein was co-expressed with folding chaperones (Ex. 33). As observed with the recombinant BotB protein (Example 40 below), the folding chaperones co-eluted with the recombinant BotA protein during the Ni-IDA purification step. Because the recombinant BotA and BotB proteins have similar molecular weights (about 1/10 the size of the non-reduced folding chaperone) and the imidazole step gradient strategy was unsuccessful in purifying BotB away from the folding chaperone (see Ex. 40), size exclusion chromatography was examined for the ability to purify the recombinant BotA protein away from the folding chaperones.

A column (2.5 x 24 cm) containing Sephacryl S-100 HR (Pharmacia) was poured (bed volume + 110 ml). Proteins having molecular weights greater than 100 K are expected to elute in the void volume under these conditions and smaller proteins should be retained by the beads and elute at different times, depending on their molecular weights. To maintain solubility of the purified BotA protein, the Sephacryl column was equilibrated in a buffer having the same salt concentration as the buffer used to elute the BotA protein from the IDA column (i.e., 50 mM sodium phosphate, 0.5 M NaCl, 10% glycerol; all reagents from Mallinkrodt, Chesterfield, MO).

Five milliliters of the IDA-purified recombinant BotA protein (Ex. 33) was filtered through a $0.45~\mu$ syringe filter, applied to the column and the equilibration buffer was pumped through the column at a flow rate of 1 ml/minute. Eluted proteins were monitored by absorbance at 280 nm and collected either manually or with a fraction collector (BioRad). Appropriate fractions were pooled, if necessary, and the protein was quantitated by absorbance at 280 nm and/or BCA protein assay (Pierce). The isolated peaks were then analyzed by native and/or SDS-PAGE to identify the proteins present and to evaluate purity. The folding chaperone eluted first, followed by the recombinant BotA protein and then the imidazole peak.

SDS-PAGE analysis (12.5% polyacrylamide, reduced samples) was used to evaluate the purity of the IDA-purified recombinant BotA protein before and after S-100 purification.

Figure 33 shows the difference in purity before and after the S-100 purification step. In Figure 33, lane 1 contains molecular weight markers (BioRad broad range). Lane 2 shows the IDA-purified recombinant BotA protein preparation, which is contaminated with significant amounts of the folding chaperone. Following S-100 purification, the amount of folding chaperone present in the BotA sample is reduced dramatically (lane 3). Lane 4 contains no protein (i.e., it is a blank lane): lanes 5-8 contain samples of IDA-purified recombinant BotB and BotE proteins and are discussed infra.

Endotoxin levels in the S-100 purified BotA preparation were determined using the LAL assay (Associates of Cape Cod) as describe in Example 24. The purified BotA preparation was found to contain 22.7 to 45.5 EU/mg recombinant protein.

These results demonstrate that size exclusion chromatography was successful in purifying the recombinant BotA protein from folding chaperones and imidazole following an initial IDA purification step. Furthermore, these results demonstrate that the S-100 purified BotA protein was substantially free of endotoxin.

15

10

5

EXAMPLE 35

Cloning And Expression Of The C Fragment Of The C. botulinum Serotype B Toxin Gene

20

25

The C. hotulinum type B neurotoxin gene has been cloned and sequenced [Whelan et al. (1992) Appl. Environ. Microbiol. 58:2345 and Hutson et al. (1994) Curr. Microbiol. 28:101]. The nucleotide sequence of the toxin gene derived from the Eklund 17B strain (ATCC 25765) is available from the EMBL/GenBank sequence data banks under the accession number X71343; the nucleotide sequence of the coding region is listed in SEQ ID NO:39. The amino acid sequence of the C. hotulinum type B neurotoxin derived from the strain Eklund 17B is listed in SEQ ID NO:40. The nucleotide sequence of the C. hotulinum serotype B toxin gene derived from the Danish strain is listed in SEQ ID NO:41 and the corresponding amino acid sequence is listed in SEQ ID NO:42.

30

The DNA sequence encoding the native *C. hotulinum* serotype B C fragment gene derived from the Eklund 17B strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:43 and the corresponding amino acid sequence is listed in SEQ ID NO:44. The DNA sequence encoding the native *C. hotulinum* scrotype B C fragment gene derived from the Danish strain can be expressed using the pETHisb vector; the

resulting coding region is listed in SEQ ID NO:45 and the corresponding amino acid sequence is listed in SEQ ID NO:46. The C fregament region from any strain of C. botulinum serotype B can be amplified and expressed using the approach illustrated below using the C fragment derived from C. botulinum type B 2017 strain.

5

The C. botulinum type B neurotoxin gene is synthesized as a single polypeptide chain which is processed to form a dimer composed of a light and a heavy chain linked via disulfide bonds; the type B neurotoxin has been reported to exist as a mixture of predominatly single chain with some double chain (Whelan et al., supra). The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H_C domain. Expression of the C fragment of C. botulinum type B toxin in heterologous hosts (e.g., E. coli) has not been previously reported.

The native C fragment of the C. botulinum serotype B toxin gene was cloned and expression constructs were made to facilitate protein expression in E. coli. This example involved PCR amplification of the gene, cloning, and construction of expression vectors.

15

10

The C fragment of the C. hotulinum serotype B (BotB) toxin gene was cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. The C. hotulinum type B 2017 strain was obtained from the American Type Culture Collection (ATCC #17843). The following primer pair was used to amplify the BotB gene: 5'-CGCCATGGCTGATACAATACTAATAGAA ATG-3' [5' primer, engineered Ncol site underlined (SEQ ID NO:47)] and 5'-GCAAG CTTTTATTCAGTCCACCCTTCATC-3' [3' primer, engineered HindIII site underlined, native gene termination codon italicized (SEQ ID NO:48)]. After cloning into the pCRscript vector, the NhcI(filled)/HindIII fragment was cloned into pETHisb vector as described for BotA C fragment gene in Example 28. The resulting construct was termed pHisBotB.

25

30

20

pHisBotB expresses the BotB gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotB expression construct was transformed into BL21(DE3) pLysS competent cells and 1 liter cultures were grown, induced and his-tagged proteins were purified utilizing a NiNTA resin (cluted in low pH elution buffer) as described in Example 28. Total, soluble and purified proteins were resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a chicken anti-C. botulinum serotype B toxoid primary antibody (generated by immunization of hens using C. botulinum serotype B toxoid as described in Example 3). Samples of BotA and BotE C fragment proteins were included on

the gels for MW and immunogenicity comparisons. Strong immunoreactivity to only the BotB protein was detected with the anti-C. botulinum serotype B toxoid antibodies. The recombinant BotB protein was expressed at low levels (3 mg/liter) as a soluble protein. The purified BotB protein migrated as a single band of the predicted MW (i.e., -50kD).

5

These results demonstrate the cloning of the native *C. hotulinum* serotype B C fragment gene, the expression and purification of the recombinant BotB protein as a soluble his-tagged protein in *E. coli*.

EXAMPLE 36

10

Generation Of Neutralizing Antibodies Using The Recombinant pHisBotB Protein

The ability of the purified pHisBot protein to generate neutralizing antibodies was examined. Nine BALBe mice were immunized with BotB protein (purified as described in Ex. 35) using Gerbu GMDP adjuvant (CC Biotech). The low pH elution was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received a subcutaneous injection of 100 µl antigen/adjuvant mix (15 µg antigen + 1 µg adjuvant) on day 0. Mice were subcutaneously boosted as above on day 14 and bled on day 28. Mice were subsequently boosted 1-2 weeks after bleeding and were then bled on day 70.

20

15

Anti-C. hotulinum serotype B toxoid titers were determined in day 28 serum from individual mice from each group using the ELISA protocol outlined in Example 29 with the exception that the plates were coated with C. hotulinum serotype B toxoid, and the primary antibody was a chicken anti-C. hotulinum serotype B toxoid. Seroconversion [relative to control mice immunized with pHisBotE antigen (described below)] was observed with all 9 mice immunized with the purified pHisBotB protein.

25

30

The ability of the anti-BotB antibodies to neutralize native *C. hotulinum* type B toxin was tested in a mouse-*C. hotulinum* neutralization model using pooled mouse serum (see Ex. 23b). The LD_{so} of purified *C. hotulinum* type B toxin complex (Dr. Eric Johnson, University of Wisconsin, Madison) was determined by a intraperitoneal (IP) method [Schantz and Kautler (1978), *supra*] using 18-22 g female ICR mice. The amount of neutralizing antibodies present in the serum of the immunized mice was determined using serum antibody titrations. The various serum dilutions (0.01 ml) were mixed with 5 LD_{so} units of *C. hotulinum* type B toxin and the mixtures were injected IP into mice. The neutralizations were performed in duplicate. The mice were then observed for signs of botulism for 4 days. Undiluted serum (day 28 or

day 70) was found to protect 100% of the injected mice while the 1:10 diluted serum did not. This corresponds to a neutralization titer of 0.05-0.5 IU/ml.

These results demonstrate that seroconversion occurred and neutralizing antibodies were induced when the pHisBotB protein was utilized as the immunogen.

. 5

EXAMPLE 37

Construction Of Vectors To Facilitate Expression Of His-Tagged BotB Protein In Fermentation Cultures

10

A number of expression vectors were constructed to facilitate the expression of recombinant BotB protein in large scale fermentation culture. These constructs varied as to the strength of the promoter utilized (T7 or T7lac) and the presence of repressor elements (laclq) on the plasmid. The resulting constructs varied in the level of expression achieved and in plasmid stability which facilitated the selection of a optimal expression system for fermentation scaleup.

15

The BotB expression vectors created for fermentation culture were engineered to utilize the kanamycin rather than the ampicillin resistance gene, and contained either the T7 or T7lac promoter, with or without the laclq gene for the reasons outlined in Example 30.

20

In all cases, the protein expressed by the various expression vectors is the pHisBot B protein described in Example 35, with the only differences between clones being the alteration of various regulatory elements. Using the designations outlined below, the pHisBotB clone (Ex. 35) is equivalent to pHisBotB amp T7lac.

25

a) Construction Of pHisBotB kan T7lac

pHisBotB kan T7lac was constructed by insertion of the *BglII/HindIII* fragment of pHisBotB which contains the BotB gene sequences into the pPA1870-2680 kan T7lac vector which had been digested with *BglII* and *HindIII* (the pPA1870-2680 kan T7lac vector contains the pET24 kan gene in the pET23 vector, such that no laclq gene is present). Proper construction of pHisBotB kan T7lac was confirmed by restriction digestion.

30

b) Construction Of pHisBotB kan laclq T7lac

pHisBotB kan lacIq T7lac was constructed by insertion of the Bg/II/HindIII fragment of pHisBotB which contains the BotB gene sequences into similarly cut pET24a vector. Proper construction of pHisBotB kan lacIq T7lac was confirmed by restriction digestion.

5

c) Construction Of pHisBotB kan laclq T7

pHisBotB kan laclq T7 was constructed by inserting the Ndel/Xhol fragment from pHisBotE kan laclq T7lac which contains the BotB gene sequences into similarly cleaved pPA1870-2680 kan laclq T7 vector (this vector contains the T7 promoter, the same N-terminal his-tag as the Bot constructs, the *C. difficile* toxin A insert, and the kan laclq genes: this cloning replaces the *C. difficile* toxin A insert with the BotB insert). Proper construction was confirmed by restriction digestion.

Expression of recombinant BotB protein from these expression vectors and purification of the BotB protein is described in Example 38 below.

15

10

EXAMPLE 38

Fermentation And Purification Of Recombinant BotB Protein Utilizing The pHisBotB kan laclq T7lac, pHisBotB kan T7lac And pHisBotB kan laclq T7 Vectors

20

The pHisBotB kan laclq T7lac, pHisBotB kan T7lac and BotB kan laclq T7 constructs [all transformed into the Bl21(DE3) strain] were grown in fermentation cultures to determine the utility of the various constructs for large scale expression and purification of soluble BotB protein. All fermentations were performed as described in Example 31.

25

30

a) Fermentation Of pHisBotB kan laclq T7lac/Bl21(DE3) Cells

The fermentation culture was induced 45 min post start of glucose feed with 1 gm IPTG (final concentration = 0.4 mM). pH was maintained at 6.5 rather than 7.0. The OD₆₀₀ was 27 at time of induction, then 35, 38, and 40 at 1-3 hrs post induction. Duplicate platings of diluted 1 hr induction samples (dilutions were prepared as described Ex. 31, dilution 3 utilized 3 μ l of dilution 2 cells) on TSA and LB+kan plates yielded 89 TSA colonies and 81 kan colonies (90% kan resistant).

Total and soluble protein extracts were resolved on a 12.5% SDS-PAGE gel and total protein was detected by staining with Coomassic blue. Low level induction of insoluble Bot

B protein was observed, increasing from 1 to 3 hrs post induction (no expression was detected in uninduced cells).

b) Fermentation Of pHisBotB kan T7lac/Bl21(DE3) Cells

- 5

10

15

20

25

30

The fermentation culture was induced 1 hr post start of glucose feed with 2 gm IPTG (final concentration = 0.8 mM). pH was maintained at 6.5 rather than 7.0. The OD_{600} was 24.5 at time of induction, then 31.5, 32, and 33 at 1-3 hrs post induction, respectively. Duplicate platings of diluted 0 hr and 2 hr induction samples (dilutions were prepared as described Ex. 31; dilution 3 utilized 3 μ l of dilution 2 cells) on TSA and LB+kan plates yielded 32 TSA colonies and 54 kan colonies (all kan resistant) for uninduced cells, and 1 TSA colony and 0 kan colonies 2 hr post induction. These results were indicative of strong induction, since yiable counts decreased dramatically 2 hrs post induction.

Total and soluble extracts were resolved on a 10% SDS-PAGE gel and total protein was detected by staining with Coomassic blue. Moderate induction of insoluble BotB protein was observed, increasing from 1 to 3 hrs post induction (no expression was detected in uninduced cells).

c) Fermentation Of pHisBotB kan laclq T7/Bl21(DE3) Cells

The fermentation was induced 2 hr post start of glucose feed with 4 gm IPTG (final concentration = 1.6 mM). pH was maintained at 6.5 rather than 7.0. The OD₈₀₀ was 45 at time of induction, then 47, 50, and 50 and 55 at 1-4 hrs post induction, respectively. Viable colony counts decreased after induction (96, 1, 1, 2, 3; dilution 3 utilized 3 μ l of dilution 2 cells). Of 63 colonies scored at the time of induction, all 63 retaining the BotB plasmid (kan resistant) and no colonies at induction grew on IPTG + Kan plates (no mutations detected).

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and total protein was detected by staining with Coomassie blue. Moderate level induction of insoluble BotB protein was observed, increasing from 1 to 4 hrs post induction (lower level expression was detected in uninduced cells, since the T7 rather than T7lac promoter was utilized).

d) Purification Of pHisBotB Protein From pHisBotB amp T7lac/Bl21(DE3) Cells

Soluble recombinant BotB protein was purified utilizing NiNTA resin from 80 ml of cell lysate generated from cells harvested from a pHisBotB fermentation [using the pHisBotB

amp T7lac/Bl21(DE3) strain]. As predicted from the small scale results above, the majority of the induced protein was insoluble. As well, the cluted material was contaminated with multiple *E. coli* contaminant proteins. A Coomassie blue-stained SDS-PAGE gel containing extracts derived from pHisBotB amp T7lac/Bl21(DE3) cells before and during purification is shown in Figure 34. In Figure 34, lane 1 contains broad range protein MW markers (BioRad). Lanes 2-5 contain extracts prepared from pHisBotB amp T7lac/Bl21(DE3) cells grown in fermentation culture: lane 2 contains total protein: lane 3 contains soluble protein: lane 4 contains protein which did not bind to the NiNTA column (*i.e.*, the flow-through) and lane 5 contains protein cluted from the NiNTA column.

10

15

20

5

Similar results were obtained using a small scale IDA column utilizing a cell lysate from the pHisBotB kan lacIq T7 fermentation described above. 250 mls of a 20% w/v PEI clarified lysate (50 gms cell pellet) of botB kan lacIq T7/Bl21(DE3) cells were purified on a small scale IDA column. The total yield of eluted protein was 21 mg protein (assuming 1 mg/ml solution = 2 OD₂₈₆/ml). When analyzed by SDS-PAGE and Coomassie staining, the BotB protein was found to comprise approximately 50% of the eluted protein with the remainder being a ladder of *E. coli* proteins similar to that observed with the NiNTA purification.

The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. The results demonstrated that a small percentage of BotB protein was soluble, that the soluble protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 1 liter fermentation harvest yielded a 67.5 gm cell pellet, this indicated that the yield of soluble affinity purified BotB protein from the IDA column was 14 mg/liter.

25

EXAMPLE 39

Co-Expression Of Recombinant BotB Proteins
And Folding Chaperones In Fermentation Cultures

30

Fermentations were performed to determine if the simultaneous overexpression of folding chaperones (*i.e.*, the Gro operon) and the BotB protein resulted in enhanced solubility of the BotB protein. This example involved fermentation of the pHisBotBkan lacIq T7lac/pACYCGro BL21(DE3), pHisBotB kan T7lac/pACYCGro Bl21(DE3) and pHisBotBkan

laclq T7/ pACYCGro BL21(DE3) cell lines. Fermentation was carried out as described in Example 31: 34 μg/ml chloramphenicol was included in the feeder and fermentation cultures.

a) Fermentation Of pHisBotBkan laclq T7lac/pACYCGro BL21(DE3) Cells

Induction was with 4 gms IPTG at 1 hr 15 min post initiation of the glucose feed. The OD₆₀₀ was 38 at time of induction, then 50, 58.5, 62 and 68 at 1-4 hrs post induction. Viable colony counts decreased during induction (24, 0, 0, 2, 0 at 0-4 hr induction; dilution 3 utilized 3 µl of dilution 2 cells). Of 24 colonies scored at the time of induction, 24 retained the BotB plasmid (kan resistant), 24 contained the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

Total and soluble extracts were resolved on 12.5% SDS-PAGE gels and were either stained with Coomassie blue or subjected to Western blotting (his-tagged proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate). This analysis revealed that the Gro chaperones were induced to high levels, but very low level expression of soluble BotB protein was observed, increasing from 1 to 4.0 hrs post induction (no expression detected in uninduced cells, induced protein detected only on Western blot). The dramatically lower expression of BotB protein in the presence of chaperone may be due to promoter occlusion (i.e., the stronger T7 promoter on the chaperone plasmid was preferentially utilized).

20

15

• 5

10

b) Fermentation Of pHisBotB kan T7lac/pACYCGro/Bl21(DE3)

Induction was with 4 gms IPTG at 1 hr post initiation of the glucose feed. The OD_{600} was 33.5 at time of induction, then 44, 51, 58.5 and 69 at 1-4 hrs post induction. Viable colony counts decreased after 2 hrs induction (43, 65, 74, 0 (70), 0 (70) at 0-4 hr induction; bracketed numbers represent microcolonies; dilution 3 utilized 3 μ l of dilution 2 cells). Most colonies at induction retained the BotB plasmid (kan resistant)and the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

30

25

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and subjected to Western blotting; his-tagged proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate. This analysis revealed that the Gro chaperones were induced to high levels and

low level expression of soluble Bot B protein was observed, increasing from 1 to 4.0 hrs post induction (no expression detected in uninduced cells).

A small scale IDA purification of BotB protein from a 250 ml PEI clarified 15% w/v extract (37.5 gm cell pellet) yielded approximately 12.5 mg protein, of which approximately 50% was BotB protein and 50% was GroEL chaperone (assessed by Coomassie staining of a 10% SDS-PAGE gel). The NiNTA alkaline phosphatase conjugate was utilized to detect histagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. The results demonstrated that all of the BotB protein produced by the pHisBotB kan T7lac/pACYCGro/Bl21(DE3) cells was soluble; the BotB protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 1 liter fermentation harvest yielded a 75 gm cell pellet, this indicated that the yield of soluble affinity purified bot B protein from this fermentation was 12.5 mg/liter. These results also demonstrated that additional purification steps are necessary to separate the chaperone proteins from the BotB

c) Fermentation Of pHisBotBkan laclq T7/pACYCGro/BL21(DE3) Cells

5

10

15

20

25

30

protein.

Induction was with 4 gms IPTG at 2 hr post initiation of the glucose feed. The OD_{600} was 46 at time of induction, then 56, 63, 69 and 71.5 at 1-4 hrs post induction. Viable colony counts decreased after induction (58, 3(5), 3, 0, 0 at 0-4 hr induction; bracketed numbers represent microcolonies; dilution 3 utilized 3 μ l of dilution 2 cells). All (53/53) colonies scored at the time of induction retained the BotB plasmid (kan resistant) and the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

Total and soluble extracts were resolved on a 10% SDS-PAGE gels and Western blotted and his-tagged proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate. This analysis revealed that the Gro chaperones were induced to high levels (observed by ponceau S staining), and a much higher expression of soluble Bot B protein (compared to expression in the pHisBotB kan T7lac/pACYCGro fermentation) was observed at all timepoints, including uninduced cells (some increase in BotB protein levels were observed after induction).

A small scale IDA purification of BotB protein from a 100 ml PEI clarified 15% w/v extract (15 gm cell pellet) yielded approximately 40 mg protein, of which approximately 50% was BotB protein and 50% was GroEL chaperone, as assessed by Coomassie staining of a 10% SDS-PAGE gel. The NiNTA alkaline phosphatase conjugate was utilized to detect histagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. The results demonstrated that a significant percentage (i.e., ~10-20 %) of BotB protein was soluble, that the solubilized protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 10 liter fermentation yielded a 108 gm cell pellet, this indicated that the yield of soluble affinity purified BotB protein from this fermentation was 144 mg/liter.

In a scale up experiment, 2 liters of a 20% w/v PEI clarified lysate of pHisBotB kan laclq T7/pACYCGro/BL21(DE3) cells were purified on a large scale IDA column. The purification was performed in duplicate. The total yield of BotB protein was 220 and 325 mgs protein in the two experiments (assuming 1 mg/ml solution = 2.0 OD₂₈₀/ml). This represents 0.7% or 1.0%, respectively, of the total soluble cellular protein (assuming a PEI lystate having a concentration of 8 mg protein/ml and that the eluted material comprises a 1:1 mixture of BotB and folding chaperone). The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. These results demonstrated that a significant percentage (i.e., ~10-20 %) of the BotB protein was soluble, that the solubilized protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 1 liter fermentation harvest yielded a 108 gm cell pellet, this indicated that the yield of soluble affinity purified BotB protein from the large scale purification was 60 mg or 89 mg/liter. These results also demonstrated that further purification would be necessary to remove the contaminating chaperone protein.

The above results provide methodologies for the purification of soluble BotB protein from fermentation cultures, in a form contaminated predominantly with a single *E. coli* protein (the folding chaperone utilized to enhance solubility). In the next example, methods are provided for the removal of the contaminating chaperone protein.

- 5

10

15

20

25

EXAMPLE 40

Removal Of Contaminating Folding Chaperone Protein From Purified Recombinant C. botulinum Type B Protein

In this example size exclusion chromatography and ultrafiltration was used to purify recombinant BotB protein from the folding chaperones and imidazole in IDA-purified material.

5 .

10

15

20

25

30

To enhance the solubility of the recombinant BotB protein during scale-up, the protein was co-expressed with folding chaperones (see Ex. 39). During the Ni-IDA purification step, the folding chaperones co-eluted with the BotB protein in 800 mM imidazole; therefore, a second purification step was required to isolate the BotB free of folding chaperones. Lane 3 of Figure 35 contains proteins eluted from an IDA column to which a lysate of pHisBotB kan laclq T7/pACYCGro/BL21(DE3) cells had been applied; the proteins were resolved on a 4-15% polyacrylamide pre-cast gradient gel (Bio-Rad, Hercules, CA) run under native conditions and then stained with Coomassie blue. In Figure 35, lanes 1 and 4 contain proteins present in peak 1 and peak 2 from a Sephacryl S-100 column run as described below; lane 2 is blank.

As seen in lane 3 of Figure 35, the IDA-purified sample consists primarily of the folding chaperones and the BotB protein. The fact that the chaperones and the BotB antigen appear as two distinct bands under native conditions suggested they were not complexed together and therefore, it should be possible to separate them, using either a gradient of imidazole concentrations or size exclusion methods.

In order to determine whether a gradient of imidazole concentrations could be used to separate the chaperone from the BotB protein, a step gradient using imidazole at 200, 400, 600, and 800 mM in 50 mM sodium phosphate, 0.5 M NaCl and 10 % glycerol, pH 6.8 was applied to an IDA column (containing proteins bound from a lysate of pHisBotB kan laclq T7/pACYCGro/BL21(DE3) cells). By narrowing the range of imidazole concentrations, it was hoped that the BotB and chaperone proteins would differentially elute at different concentrations of imidazole. Eluted proteins were monitored by absorbance at 280 nm and collected either manually or with a fraction collector (BioRad). Protein was found to elute at 200 and 400 mM imidazole only.

Figure 36 shows a Coomassie stained SDS-PAGE gel containing protein eluted during the imidazole step gradient. Lane 1 contains broad range MW markers (BioRad). Lane 2

contains BotB protein purified by IDA chromatography of an extract of pHisBotB/BL21(DE3) pLysS cells grown in shaker flask culture (i.e., no co-expression of chaperones: Ex. 35). Lane 3 contains a 20% w/v PEI clarified lysate of pHisBotB kan laclq T7/pACYCGro/BL21(DE3) cells (i.e., the lysate prior to purification by IDA chromatography). Lanes 4 and 5 contain protein which eluted at 200 or 400 mM imidazole, respectively. Lane 6 is blank. Lanes 7 and 8 contain 1/5 the load present in lanes 4 and 5.

As shown in Figure 36, both the chaperone and the BotB protein eluted in 200 mM imidazole, and more chaperone elutes in 400 mM imidazole, however no concentration of imidazole tested permitted the elution of BotB protein alone. Consequently, no significant purification was achieved using imidazole at these concentrations.

Because of the considerable difference in molecular weights between the folding chaperone, which is a multimer with a total molecular weight around 400 kD (as determined on a Shodex KB 804 sizing column by HPLC), and the recombinant BotB protein (molecular weight around 50 kD), size exclusion chromatography was next examined for the ability to separate these proteins.

a) Size Exclusion Chromatography

- 5

10

15

20

25

30

A column containing Sephacryl S-100 HR (S-100) (Pharmacia) was poured (2.5 cm x 24 cm; ~110 ml bed volume). The column was equilibrated in a buffer consisting of phosphate buffered saline (10mM potassium phosphate, 150 mM NaCl, pH 7.2) and 10 % glycerol (Mallinkrodt). Typically, 5 ml of the IDA-purified BotB protein was filtered through a $0.45~\mu$ syringe filter and applied to the column, and the equilibration buffer was pumped through the column at a flow rate of 1 ml/minute. Eluted proteins were monitored by absorbance at 280 nm and collected either manually or with a fraction collector. Appropriate tubes were pooled, if necessary, and the protein was quantitated by absorbance at 280 nm and/or by BCA protein assay. The isolated peaks were then analyzed by native and/or SDS-PAGE to identify the protein and evaluate the purity.

Because of its larger size, the folding chaperone eluted first, followed by the recombinant BotB protein. A smaller third peak was observed which failed to stain when analyzed by SDS-PAGE and therefore was presumed to be imidazole.

SDS-PAGE analysis (12.5% polyacrylamide, reduced samples) was used to evaluate the purity of the IDA-purified recombinant BotB protein before and after S-100 purification. The results are shown in Figure 33.

In Figure 33, lane 1 contains broad range MW markers (BioRad). Lane 5 contains IDA-purified BotB protein. Lane 6 contains IDA-purified BotB protein following S-100 purification. Lane 7 is blank (lanes 2-4 were discussed in Ex. 34 above).

5

10

15

20

25

30

The results shown in Figure 33 show that the IDA-purified BotB is significantly contaminated with the folding chaperone (molecular weight about 60 kD under reducing conditions: lane 6). Following S-100 purification, the amount of folding chaperone present in the BotB sample was reduced dramatically (lane 7). Visual inspection of the Coomassie stained SDS-PAGE gel revealed that after S-100 purification, > 90% of the total protein present was BotB.

The IDA-purified BotB and the S-100-purified BotB samples were analyzed by HPLC on a size exclusion column (Shodex KB 804); this analysis revealed that the BotB protein represented 64% of the total protein in the IDA-purified sample and that following S-100 purification, the BotB protein represented >95% of the total protein in the sample.

The IDA-purified BotB material was also applied to a ACA 44 (SpectraPor, Houston, TX) column. The ACA 44 resin is equivalent to the S-100 resin and chromatography using the ACA 44 resin was carried out exactly as described above for the S-100 resin. The ACA 44 resin was found to separate the recombinant BotB protein from the folding chaperone. The ACA 44-purified BotB sample was analyzed for endotoxin using the LAL assay (Associates of Cape Cod) as describe in Example 24. Two aliquouts of the ACA 44-purified BotB preparation were analyzed and were found to contain either 58 to 116 EU/mg recombinant protein or 94 to 189 EU/mg recombinant protein.

These results demonstrate that size exclusion chromatography can be used to purify the recombinant BotB protein from the folding chaperone and imidazole in IDA-purified material.

b) Ultrafiltration For The Separation Of Recombinant BotB Protein And Chaperones

Ultrafiltration was examined as an alternative method for the separation recombinant BotB protein and folding chaperones in IDA-purified material. While in this example only mixtures of BotB and chaperones were separated by ultrafiltration, this technique is suitable for use with recombinant BotA and BotE proteins as well provided that the wash buffers used are altered as necessary to take into account different requirements for solubility.

The recombinant BotB protein and folding chaperones were separated using a two-step sequential ultrafiltration method. The first membrane used had a nominal molecular weight

cutoff (MWCO) of approximately 100 kD; this membrane retains the larger folding chaperone while allowing the smaller recombinant protein to pass through. The addition of several volumes of wash buffer may be required to efficiently wash the recombinant protein through the membrane. The second step utilized a membrane with a nominal MWCO of approximately 10 kD. During this step, the recombinant antigen was retained by the membrane and could be concentrated to the degree desired and the imidazole and excess wash buffer passed through the membrane.

- 5

10

15

20

25

30

Twenty-seven milliliters of an IDA-purified BotB preparation was ultrafiltered through a 47 mm YM 100 (100 kD MWCO) membrane (Amicon) in a 50 ml stirred cell (Amicon). The membrane was washed in dd H₂O prior to use as recommended by the manufacturer. Six volumes of 10% glycerol in PBS were washed through to remove most of the recombinant BotB protein and this wash was collected in a separate vessel. The resulting BotB protein-rich filtrate was then concentrated 12-fold using a YM 10 (10 kD MWCO) membrane (Amicon), to a final volume of 14 ml. The YM 100 and YM 10 concentrates were analyzed along with the lysate starting material by native PAGE using a 4-15% pre-cast gradient gel (BioRad). The results are shown in Figure 37.

In Figure 37, lane 1 contains IDA-purified BotB derived from a shaker flask culture (i.e., no co-expression of chaperones; Ex. 35); lane 2 contains a 20% w/v PEI clarified lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells; lane 3 shows the lysate of lane 3 after IDA purification; lane 4 contains the YM 10 concentrate and lane 5 contains the YM 100 concentrate.

The results shown in Figure 37 demonstrate that the recombinant BotB protein can be purified away from the folding chaperone by ultrafiltration through a 100 kD MWCO membrane (lane 4), leaving the chaperone protein in the 100 kD concentrate (lane 5). Analysis of the sample in lane 5 also showed that very little of the BotB protein was retained by the 100 kD MWCO membrane after 6 volumes of wash buffer had been applied.

The BotB samples following IDA chromatography and following ultrafiltration through the YM 100 membrane were analyzed by HPLC on a size exclusion column (Shodex KB 804): this analysis revealed that the BotB protein represented 64% of the total protein in the IDA-purified sample and that following ultrafiltration through the YM 100 membrane, the BotB protein represented >96% of the total protein in the sample.

The BotB protein purified by ultrafiltration through the YM 100 membrane was examined for endotoxin using the LAL assay (Associates of Cape Cod) as describe in

Example 24. Two aliquouts of the YM 100-purified BotB preparation were analyzed and were found to contain either 18 to 36 EU/mg recombinant protein or 125 to 250 EU/mg recombinant protein.

The above results demonstrate that size exclusion chromatography and ultrafiltration can be used to purify recombinant botulinal toxin proteins away from folding chaperones.

EXAMPLE 41

Cloning And Expression Of The C Fragment Of The C. botulinum Serotype E Toxin Gene

10

15

20

5

The C. hotulinum type E neurotoxin gene has been cloned and sequenced from several different strains [Poulet et al. (1992) Biochem. Biophys. Res. Commun. 183:107 (strain Beluga): Whelan et al. (1992) Eur. J. Biochem. 204:657 (strain NCTC 11219): Fujii et al. (1990) Microbiol. Immunol. 34:1041 (partial sequence of strains Mashike, Iwani and Otaru) and Fujii et al. (1993) J. Gen. Microbiol. 139:79 (strain Mashike)]. The nucleotide sequence of the type E toxin gene is available from the EMBL sequence data bank under accession numbers X62089 (strain Beluga) and X62683 (strain NCTC 11219). The nucleotide sequence of the coding region (strain Beluga) is listed in SEQ ID NO:49. The amino acid sequence of the C. hotulinum type E neurotoxin derived from strain Belgua is listed in SEQ ID NO:50. The nucleotide sequence of the coding region (strain NCTC 11219) is listed in SEQ ID NO:51. The amino acid sequence of the C. hotulinum type E neurotoxin derived from strain NCTC 11219 is listed in SEQ ID NO:52.

25 p
a
f

30

The DNA sequence encoding the native *C. hotulinum* serotype E. C. fragment gene derived from the Beluga strain can be expressed as a histidine-tagged protein using the pETHisb vector: the resulting coding region is listed in SEQ ID NO:53 and the corresponding amino acid sequence is listed in SEQ ID NO:54. The DNA sequence encoding the *C* fragment of the native *C. hotulinum* serotype E. gene derived from the NCTC 11219 strain can be expressed as a histidine-tagged fusion protein using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:55 and the corresponding amino acid sequence is listed in SEQ ID NO:56. The *C* fragment region from any strain of *C. hotulinum* serotype E can be amplified and expressed using the approach illustrated below using the *C* fragment derived from *C. hotulinum* type E 2231strain (ATCC #17786).

The type E neurotoxin gene is synthesized as a single polypeptide chain which may be converted to a double-chain form (i.e., a heavy chain and a light chain) by cleavage with trypsin: unlike the type A neurotoxin, the type E neurotoxin exists essentially only in the single-chain form. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H_C domain. Expression of the C fragment of C hotulinum type E toxin in heterologous hosts (e.g., E, coli) has not been previously reported.

1.5

10

15

20

25

30

The native C fragment of the C. botulinum serotype E toxin (BotE) gene was cloned and inserted into expression vectors to facilitate expression of the recombinant BotE protein in E. coli. This example involved PCR amplification of the gene, cloning, and construction of expression vectors.

The BotE serotype gene was isolated using PCR as described for the BotA serotype gene in Example 28. The *C. botulinum* type E strain was obtained from the American Type Culture Collection (ATCC #17786: strain 2231). The following primer pair was used in the PCR amplification: 5'-CGCCATGGCTCTTTCTTCTTAT ACAGATGAT-3' (5' primer, engineered *Ncol* site underlined) (SEQ ID NO:57) and 5'-GCAAGCTT774TTTTTCTTGCCATCCATG-3' (3' primer, engineered *HindIII* site underlined, native gene termination codon italicized) (SEQ ID NO:58). The PCR product was inserted into pCRscript as described in Example 28. The resulting pCRscript BotE clone was confirmed by restriction digestion, as well as, by obtaining the sequence of approximately 300

bases located at the 5' end of the C fragment coding region using standard DNA sequencing methods. The resulting BotE sequence was identical to that of the published C. botulinum type E toxin sequence [Whelan et al (1992), supra].

The Nhel(filled)/HindIII fragment from a pCRscript BotE recombinant was cloned into pETHisb vector as described for BotA C fragment in Example 28. The resulting construct was termed pHisBotE. pHisBotE expresses the BotE gene under the control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag.

The pHisBotE expression construct was transformed into BL21(DE3) pLysS competent cells and 1 liter cultures were grown, induced and his-tagged proteins were purified utilizing a NiNTA resin (eluted in low pH elution buffer) as described in Example 28. Total, soluble and purified proteins were resolved by SDS-PAGE and detected by Coomassic staining. The results are shown in Figure 38.

In Figure 38, lane 1 contains broad range MW markers (BioRad): lane 2 contains a total protein extract: lane 3 contains a soluble protein extract: lane 4 contains proteins present

in the flow through from the NiNTA column (this sample was not diluted prior to loading and therefore represents a load 5X that of the load applied for the total and soluble extracts in lanes 2 and 3); lane 5 contains proteins eluted from the NiNTA column; lane 6 contains protein eluted from a NiNTA column which had been stored at -20°C for 1 year.

5

The pHisBotE protein was expressed at moderate levels (7 mg/liter) as a totally soluble protein. The purified protein migrated as a single band of the predicted MW.

10

Western blot hybridization utilizing a chicken anti-C. botulinum serotype E toxoid primary antibody (generated by immunization of hens as described in Example 3 using C. botulinum serotype E toxoid) was also performed on the total, soluble and purified BotE proteins. Samples of BotA and BotB C fragments were also included on the gels to facilitate MW and immunogenicity comparisons. Strong immunoreactivity was detected using the anti-C. botulinum type E toxoid antibody only with the BotE protein.

These results demonstrate that the native BotE gene sequences can be expressed as a soluble his-tagged protein in *E. coli* and purified by metal-chelation affinity chromatography.

15

EXAMPLE 42

Generation Of Neutralizing Antibodies Using The Recombinant pHisBotE Protein

20

The ability of the purified pHisBotE protein to generate neutralizing antibodies was examined. Nine BALBc mice were immunized with BotE protein (purified as described in Ex. 41) using Gerbu GMDP adjuvant (CC Biotech). The low pH elution was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received a subcutaneous injection of 100 µl antigen/adjuvant mix (35 µg antigen + 1 µg adjuvant) on day 0. Mice were subcutaneously boosted as above on day 14 and bled on day 28. Mice were subsequently boosted and bled on day 70.

25

Anti-C. hotulinum serotype E toxoid titers were determined in day 28 serum from individual mice from each group using the ELISA protocol outlined in Example 29 with the exception that the plates were coated with C. hotulinum serotype E toxoid, and the primary antibody was a chicken anti-C. hotulinum serotype E toxoid. Seroconversion [relative to control mice immunized with the p6xHisBotA antigen (Ex. 29)] was observed with all 9 mice immunized with the purified pHisBotE protein.

30

The ability of the anti-BotE antibodies to neutralize native C. botulinum type E toxin was tested in a mouse-C. botulinum neutralization model using pooled mouse serum (see Ex.

23b). The LD₅₀ of purified *C. hotulinum* type E toxin complex (Dr. Eric Johnson, University of Wisconsin, Madison) was determined by a intraperitoneal (IP) method [Schantz and Kautler (1978), supra] using 18-22 g female ICR mice. The amount of neutralizing antibodies present in the serum of the immunized mice was determined using serum antibody titrations. The various serum dilutions (0.01 ml) were mixed with 5 LD₅₀ units of *C. hotulinum* type E toxin and the mixtures were injected IP into mice. The neutralizations were performed in duplicate. The mice were then observed for signs of botulism for 4 days. Undiluted serum from day 28 did not protect, while undiluted, 1/10 diluted and 1/100 diluted day 70 serum protected (1005 of animals) while 1/1000 diluted day 70 serum did not. This corresponds to a neutralization titer of 50-500 IU/ml.

These results demonstrate that seroconversion occurred and neutralizing antibodies were induced when the recombinant BotE protein was utilized as the immunogen.

EXAMPLE 43

Construction Of Vectors To Facilitate Expression
Of His-Tagged BotE Protein In Fermentation Cultures

A number of expression vectors were constructed to facilitate the expression of recombinant BotE protein in large scale fermentation culture. These constructs varied as to the strength of the promoter utilized (T7 or T7lac) and the presence of repressor elements (laclq) on the plasmid. The resulting constructs varied in the level of expression achieved and in plasmid stability which facilitated the selection of a optimal expression system for fermentation scaleup. This example involved a) construction of BotE expression vectors and b) determination of expression levels in small scale cultures.

25

20

• 5

10

15

a) Construction Of BotE Expression Vectors

The BotE expression vectors created for fermentation culture were engineered to utilize the kanamycin rather than the ampicillin resistance gene, and contained either the T7 or T7lac promoter, with or without the laclq gene for the reasons outlined in Example 30.

30

In all cases, the protein expressed by the various expression vectors is the pHisBotE protein described in Example 41, with the only differences between clones being the alteration of various regulatory elements. Using the designations outlined below, the pHisBotE clone (Ex. 41) is equivalent to pHisBotE amp T7lac.

i) Constructi n Of pHisBotE kan laclq T7lac

pHisBotE kan laclq T7lac was constructed by inserting the Xhal/HindIII fragment of pHisBotE which contains the BotE gene sequences into Xhal/HindIII-cleaved pET24a vector. Proper construction was confirmed by restriction digestion.

5

ii) Construction Of pHisBotE kan T7

pHisBotE kan T7 was constructed by ligating the BotE-containing Xbal/Sapl fragment of pHisBotE kan laclqT7lac to the T7 promoter-containing Xbal/Sapl fragment of pET23a. Proper construction was confirmed by restriction digestion.

10

iii) Construction Of pHisBotE kan laclgT7

pHisBotE kan laclqT7 was constructed by inserting the Bg/II/HindIII fragment from pHisBotE kan T7 which contains the BotE gene sequences into Bg/II/HindIII-cleaved pET24 vector. Proper construction was confirmed by restriction digestion.

15

20

b) Determination Of BotE Expression Levels In Small Scale Cultures

The three BotE kan expression vectors described above were transformed into BI21(DE3) competent cells and 50 ml (2XYT + 40 µg/ml kan) cultures were grown and induced with ITPG as described in Example 28. Total and soluble protein extracts from before and after induction made as described in Example 28. The total and soluble extracts were resolved on a 12.5% SDS-PAGE gel, and his-tagged proteins were detected on a Western blot utilizing the NiNTA-alkaline phosphatase conjugate as described in Example 31(c)(iii). The results showed that all three BotE cell lines expressed his-tagged proteins of the predicted MW for the BotE protein upon induction. The results also demonstrated that the two constructs that contained the T7 promoter expressed the BotE protein before induction, while the T7lac promoter construct did not. Upon induction, the T7 promoter-containing constructs induced to higher levels than the T7lac-containing construct, with the pHisBotE kan laclqT7/Bl21(DE3) cells accumulating the maximal levels of BotE protein.

30

25

EXAMPLE 44

Expression And Purification Of pHisBotE From Fermentation Cultures

Based on the small scale inductions performed in Example 43, the pHisBotE kan lacIq T7/Bl21(DE3) strain was selected for fermentation scaleup. This example involved the fermentation and purification of recombinant BotE C fragment protein.

5

10

1.5

20

25

30

A fermentation with the pHisBotE kan laclq T7/Bl21(DE3) strain was performed as described in Example 31. The fermentation culture was induced 2 hrs post start of the glucose feed with 4 gm IPTG (final concentration = 1.6 mM). The OD₆₀₀ was 42 at time of induction, then 46.5, 48, 53 and 54 at 1-4 hrs post induction. Viable colony counts decreased from 0-4 hr induction [131, 4 (28), 7 (3), 7, 8; dilution 3 utilized 6 μl of dilution 2 cells; bracketed colonies are microcolonies]. All (32/32) colonies scored at the time of induction retained the BotE plasmid (kan resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected). These results were indicative of strong promoter induction, since colony viability reduced after induction, and the culture stopped growing during fermentation (stopped at 54 OD₆₀₀/ml).

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and total protein was detected by staining with Coomassie blue. The results are shown in Figure 39.

In Figure 39, lane 1 contains total protein from a pHisBotA kan T7 lac/Bl21(DE3) pLysS fermentation (Ex. 24). Lanes 2-9 contain extracts prepared from the above pHisBotE kan laclq T7/Bl21(DE3) fermentation; lanes 2-4 contain total protein extracts prepared at 0, 1 and 2 hours post-induction, respectively. Lane 5 contains a soluble protein extract prepared at 2 hours post-induction. Lanes 6 and 7 contain total and soluble extracts prepared at 3 hours post-induction, respectively. Lanes 8 and 9 contain total and soluble extracts prepared at 4 hours post-induction, respectively. Lanes 8 and 9 contain total and soluble extracts prepared at 4 hours post-induction, respectively. Lane 10 contains broad range MW markers (BioRad).

The results shown in Figure 39 demonstrate that moderate level induction of totally soluble Bot E protein was observed, increasing from 1 to 4 hrs post induction (no expression was detected in uninduced cells). From a 2 liter fermentation harvest a 155 gm (wet wt) cell pellet was obtained and used to make a PEI-clarified lysate (1 liter in CRB, pH 6.8). The lysate was applied to a large scale IDA column and 200 mg of BotE protein, which was found to be greater than 95% pure (as judged by visual inspection of a Coomassie stained SDS-PAGE gel), was recovered. This represents 2.5% of the total soluble cellular protein

(assuming a PEI lysate having a concentration of 8 mg protein/ml) and corresponds to a yield of 100 mg BotE protein/liter of fermentation culture.

The above results demonstrate that high levels of the recombinant BotE protein can be expressed and purified from fermentation cultures.

5

10

15

EXAMPLE 45

Removal Of Imidazole From Purified Recombinant BotE Protein Preparations

The expression of recombinant BotE protein, unlike the BotA and BotB proteins, did not require the presence of folding chaperones to maintain solubility during scale-up. A size exclusion chromatography step was included however to remove the imidazole from the sample and exchange the IDA elution buffer for one consistent with the BotA antigen.

A Sephacryl S-100 FIR (S-100; Pharmacia) column was poured (2.5 cm x 24 cm; bed volume ~ 110 ml). Under these conditions, the BotE protein should be retained by the beads to a lesser degree than the smaller imidazole, therefore the BotE protein should clute from the column before the imidazole. The column was equilibrated in a buffer consisting of 50 mM sodium phosphate, 0.5 M NaCl, and 10% glycerol (all reagents from Mallinkrodt). Five milliliters of the IDA-purified BotE protein (Ex. 44) was filtered through a 0.45 μ syringe filter and applied to the S-100 column, and equilibration buffer was pumped through the column at a flow rate of 1 ml/minute. Eluted proteins were monitored by absorbance at 280 nm, and collected either manually or with a fraction collector. Appropriate tubes were pooled if necessary, and the protein was quantitated by absorbance at 280 nm and/or BCA protein assay. The isolated peaks were then analyzed by native and/or SDS-PAGE to identify the protein(s) and evaluate the purity.

25

20

Figure 40 provides a representative chromatogram showing the purification of IDA-purified BotE on the S-100 column. Even though folding chaperones were not over-expressed with this antigen, a small amount of protein eluted at a time consistent with the folding chaperones expressed with BotA and BotB proteins (Gro) (see the first peak). The second peak in the chromatogram contained the BotE protein, and the third peak was presumably imidazole. This presumed imidazole peak was isolated in comparable levels in IDA-purified BotA and BotB protein preparations as well.

30

These results demonstrate that size exclusion chromatography can be used to remove imidazole and traces of contaminating high molecular weight proteins from IDA-purified BotE protein preparations.

The S-100-purified BotE protein was tested for endotoxin contamination using the LAL assay as described in Example 24. This preparation was found to contain 64 to 128 EU/mg recombinant protein and is therefore substantially free of endotoxin.

The S-100 purified BotE was mixed with purified preparations of BotA and BotB proteins and used to immunize mice: 5 µg of each Bot protein was used per immunization and alum was included as an adjuvant. After two immunizations with this trivalent vaccine, the immunized mice were challanged with C. botulinum toxin. The immunized mice contained neutralizing antibodies sufficient to neutralize between 100.000 to 1.000.000 LD_{so} of either toxin A or toxin B and between 1.000 to 10.000 LD_{so} of toxin E. The titer of neutralizing antibodies directed against toxin E would be expected to increase following subsequent boosts with the vaccine. These results demonstrate that a trivalent vaccine containing recombinant BotA. BotB and BotE proteins provokes neutralizing antibodies.

EXAMPLE 46

Expression Of The C Fragment Of The C. hotulinum

Serotype C Toxin Gene And Generation Of Neutralizing Antibodies

20

25

30

- 5

10

15

The C. botulinum type C1 neurotoxin gene has been cloned and sequenced [Kimura et al. (1990) Biochem. Biophys. Res. Comm. 171:1304]. The nucleotide sequence of the toxin gene derived from the C. botulinum type C strain C-Stockholm is available from the EMBL/GenBank sequence data banks under the accession number D90210: the nucleotide sequence of the coding region is listed in SEQ ID NO:59. The amino acid sequence of the C. botulinum type C1 neurotoxin derived from this strain is listed in SEQ ID NO:60.

The DNA sequence encoding the native *C. botulinum* serotype C1 C fragment gene derived from the C-Stockholm strain can be expressed using the pETHisb vector: the resulting coding region is listed in SEQ ID NO:61 and the corresponding amino acid sequence is listed in SEQ ID NO:62. The C fragment region from any strain of *C. botulinum* serotype C can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type C C-Stockholm strain. Expression of the C fragment of *C. botulinum* type C1 toxin in heterologous hosts (e.g., E. coli) has not been previously reported.

The C fragment of the C. botulinum serotype C1 (BotC1) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. A number of C. botulinum serotype C strains (expressing either or both C1 and C2 toxin) are available from the ATCC [e.g., 2220 (ATCC 17782), 2239 (ATCC 17783), 2223 (ATCC 17784; a type C-β strain; C-β strains produce C2 toxin). 662 (ATCC 17849; a type C-α strain; C-α strains produce mainly C1 toxin and a small amount of C2 toxin), 2021 (ATCC 17850; a type C-α strain) and VPI 3803 (ATCC 25766)]. Alternatively, other type C strains may be employed for the isolation of sequences encoding the C fragment of C. botulinum serotype C toxin.

5

10

15

20

25

30

The following primer pair is used to amplify the BotC gene: 5'-CGCCATGGC TTTATTAAAAGATATAATTAATG-3' [5' primer, engineered Neol site underlined (SEQ ID NO:63)] and 5'-GCAAGCTTTTATTCACTTACAGGTAC AAAACC-3' [3' primer, engineered HindIII site underlined, native gene termination codon italicized (SEQ ID NO:64)]. Following PCR amplification, the PCR product is inserted into the pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as described for BotA C fragment gene in Example 28. The resulting construct is termed pHisBotC. Proper construction is confirmed by DNA sequencing of the BotC sequences contained within pHisBotC.

pHisBotC expresses the BotC gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotC expression construct is transformed into B1.21(DE3) pLysS competent cells and 1 liter cultures are grown, induced and his-tagged proteins are purified utilizing a NiNTA resin (eluted in 250 mM imidazole, 20% glycerol) as described in Example 28. Total, soluble and purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen) which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis permits the determination of expression levels of the pHisBotC protein (i.e., number of mg/liter expressed as a soluble protein). The purified BotC protein will migrate as a single band of the predicted MW (i.e., ~50kD).

The level of expression of the pHisBotC protein may be modified (increased) by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the laclq gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in fermentation cultures as described in Example 30. If only very low levels (i.e., less than 0.5%) of soluble pHisBotC protein are expressed using the above expression systems, the pHisBotC construct

may be co-expressed with pACYCGro construct as described in Example 32. In this case, the recombinant BotC protein may co-purify with the folding chaperones. The contaminating chaperones may be removed as described in Example 34. Preparations of purified pHisBotC protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotC protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotC protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotC antibodies to neutralize native C. botulinum type C toxin is demonstrated using the mouse-C. botulinum neutralization model described in Example 36.

EXAMPLE 47

Expression Of The C Fragment Of The C botulinum

Serotype D Toxin Gene And Generation Of Neutralizing Antibodies

15

20

25

30

- 5

10

The C. hotulinum type D neurotoxin gene has been cloned and sequenced [Sunagawa et al. (1992) J. Vet. Med. Sci. 54:905 and Binz et al. (1990) Nucleic Acids Res. 18:5556]. The nucleotide sequence of the toxin gene derived from the CB16 strain is available from the EMBL/GenBank sequence data banks under the accession number S49407: the nucleotide sequence of the coding region is listed in SEQ ID NO:65. The amino acid sequence of the C. hotulinum type D neurotoxin derived from the CB16 strain is listed in SEQ ID NO:66.

The DNA sequence encoding the native C. botulinum serotype D C fragment gene derived from a BotD expressing strain can be expressed using the pETHisb vector: the resulting coding region is listed in SEQ ID NO:67 and the corresponding amino acid sequence is listed in SEQ ID NO:68. The C fragment region from any strain of C. botulinum serotype D can be amplified and expressed using the approach illustrated below using the C fragment derived from C botulinum type D CB16 strain. Expression of the C fragment of C. botulinum type D toxin in heterologous hosts (e.g., E, coli) has not been previously reported.

The C fragment of the C botulinum serotype D (BotD) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. A number of C botulinum type D strains are available from the ATCC [e.g., ATCC 9633, 2023 (ATCC 17851), and VPI 5995 (ATCC 27517)].

The following primer pair is used to amplify the BotD gene: 5'-CGCCATGGC TTTATTAAAAGATATAATTAATG-3' [5' primer, engineered Ncol site underlined (SEQ ID NO:63)] and 5'-GCAAGCTTTTACTCTACCCATCCTGGATCCCT-3' [3' primer, engineered IlindIII site underlined, native gene termination codon italicized (SEQ ID NO:69)]. Following PCR amplification, the PCR product is inserted into the pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as described for BotA C fragment gene in Example 28. The resulting construct is termed pHisBotD.

5

10

15

20

25

30

pHisBotD expresses the BotD gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotD expression construct is transformed into BL21(DE3) pLysS competent cells and 1 liter cultures are grown, induced and his-tagged proteins are purified utilizing a NiNTA resin as described in Example 28. Total, soluble and purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen) which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis permits the determination of expression levels of the pHisBotD protein (i.e., number of mg/liter expressed as a soluble protein). The purified BotD protein will migrate as a single band of the predicted MW (i.e., >50kD).

The level of expression of the pHisBotD protein may be modified (increased) by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the lacIq gene on the expression plasmid, and plasmid expressed in BI.21(DE3) cell lines in fermentation cultures as described in Example 30. If only very low levels (i.e., less than about 0.5%) of soluble pHisBotD protein are expressed using the above expression systems, the pHisBotD construct may be co-expressed with pACYCGro construct as described in Example 32. In this case, the recombinant BotD protein may co-purify with the folding chaperones. The contaminating chaperones may be removed as described in Example 34. Preparations of purified pHisBotD protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotD protein is used to generate neutralizing antibodies. BALBe mice are immunized with the BotD protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotD antibodies to neutralize native C hotulinum type D toxin is demonstrated using the mouse-C hotulinum neutralization model described in Example 36.

EXAMPLE 48

Expression Of The C Fragment Of The C botulinum Serotype F Toxin Gene And Generation Of Neutralizing Antibodies

The C. botulinum type F neurotoxin gene has been cloned and sequenced [East et al. (1992) FEMS Microbiol. Lett. 96:225]. The nucleotide sequence of the toxin gene derived from the 202F strain (ATCC 23387) is available from the EMBL/GenBank sequence data banks under the accession number M92906: the nucleotide sequence of the coding region is listed in SEQ ID NO:70. The amino acid sequence of the C. botulinum type F neurotoxin derived from the 202F strain is listed in SEQ ID NO:71.

The DNA sequence encoding the native C. botulinum serotype F C fragment gene derived from the 202F strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:72 and the corresponding amino acid sequence is listed in SEQ ID NO:73. The C fragment region from any strain of C. botulinum serotype F can be amplified and expressed using the approach illustrated below using the C fragment derived from C botulinum type F 202F strain. Expression of the C fragment of C botulinum type F toxin in heterologous hosts (e.g., E. coli) has not been previously reported.

15

20

25

30

The C fragment of the C. botulinum serotype F (BotF) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. The C botulinum type F 202F strain is obtained from the American Type Culture Collection (ATCC 23387). Alternatively, sequences encoding the BotF toxin may be isolated from any BotF expressing strain [e.g., VPI 4404 (ATCC 25764), VPI 2382 (ATCC 27321) and Langeland (ATCC 35415)].

The following primer pair is used to amplify the BotF gene: 5'-CGCCATGGC
TATTCTAATTATATATTTTAATAG-3' [5' primer, engineered Ncol site underlined (SEQ ID NO:74)] and 5'-GCAAGCTTTCATTCTTTCCATCCATCCATTCTC-3' [3' primer, engineered HindIII site underlined, native gene termination codon italicized (SEQ ID NO:75)].
Following PCR amplification, the PCR product is inserted into the pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as described for BotA C fragment gene in Example 28. The resulting construct is termed pHisBotF.

pHisBotF expresses the BotF gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotF expression construct is transformed into BL21(DE3) pLysS competent cells and 1

liter cultures are grown, induced and his-tagged proteins are purified utilizing a NiNTA resin as described in Example 28. Total, soluble and purified proteins are resolved by SDS-PAGE and detected by Coomassic staining and Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen) which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis permits the determination of expression levels of the pHisBotF protein (i.e., number of mg/liter expressed as a soluble protein). The purified BotF protein will migrate as a single band of the predicted MW (i.e., ~50kD).

The level of expression of the pHisBotF protein may be modified (increased) by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the lacIq gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in fermentation cultures as described in Example 30. If only very low levels (i.e., less than about 0.5%) of soluble pHisBotF protein are expressed using the above expression systems, the pHisBotF construct may be co-expressed with pACYCGro construct as described in Example 32. In this case, the recombinant BotF protein may co-purify with the folding chaperones. The contaminating chaperones may be removed as described in Example 34. Preparations of purified pHisBotF protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotF protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotF protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotF antibodies to neutralize native C hotulinum type F toxin is demonstrated using the mouse-C hotulinum neutralization model described in Example 36.

EXAMPLE 49

25

30

20

5

10

15

Expression Of The C Fragment Of The C botulinum

Serotype G Toxin Gene And Generation Of Neutralizing Antibodies

The C. botulinum type G neurotoxin gene has been cloned and sequenced [Campbell et al. (1993) Biochimica et Biophysica Acta 1216:487 and Binz et al. (1990) Nucleic Acids Res. 18:5556]. The nucleotide sequence of the toxin gene derived from the 113/30 strain (NCFB 3012) is available from the EMBL/GenBank sequence data banks under the accession number X74162; the nucleotide sequence of the coding region is listed in SEQ ID NO:76. The amino

acid sequence of the C. hotulinum type G neurotoxin derived from this strain is listed in SEQ ID NO:77.

The DNA sequence encoding the native *C. botulinum* serotype G C fragment gene derived from the 113/30 strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:78 and the corresponding amino acid sequence is listed in SEQ ID NO:79. The C fragment region from any strain of *C. botulinum* serotype G can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type G 113/30 strain. Expression of the C fragment of *C. botulinum* type G toxin in heterologous hosts (e.g., E. coli) has not been previously reported.

- 5

10

15

20

25

30

The C fragment of the C. botulinum serotype G (BotG) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. The C. botulinum type G 113/30 strain is obtained from the NCFB. The following primer pair is used to amplify the BotG gene: 5'-CGCCATGGCTGAC ACAATTTTAATACA AGT-3' [5' primer, engineered Ncol site underlined (SEQ ID NO:80)] and 5'-GCCTCGAGTTATTCTGTCCATCCATCCTTCATCCAC-3' [3' primer, engineered Xhol site

underlined, native gene termination codon italicized (SEQ ID NO:81)]. Following PCR amplification, the PCR product is inserted into the pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as described for BotA C fragment gene in Example 28 with the exception that the sequences encoding BotG are excised from the pCRscript vector by digestion with Ncol and Xhol and the Ncol site is blunted (the BotG sequences contain an internal HindIII site). This Ncol(filled)/Xhol fragment is then ligated to the pETHisb vector which has been digested with Nhel and Sall and the Nhel site is blunted. The resulting construct is termed pHisBotG.

pHisBotG expresses the BotG gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotG expression construct is transformed into BL21(DE3) pLysS competent cells and 1 liter cultures are grown, induced and his-tagged proteins are purified utilizing a NiNTA resin as described in Example 28. Total, soluble and purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen) which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis permits the determination of expression levels of the pHisBotG protein (i.e., number of mg/liter expressed as a soluble protein). The purified BotG protein will migrate as a single band of the predicted MW (i.e., ~50kD).

The level of expression of the pHisBotG protein may be modified (increased) by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the laclq gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in fermentation cultures as described in Example 30. If only very low levels (i.e., less than about 0.5%) of soluble pHisBotG protein are expressed using the above expression systems, the pHisBotG construct may be co-expressed with pACYCGro construct as described in Example 32. In this case, the recombinant BotG protein may co-purify with the folding chaperones. The contaminating chaperones may be removed as described in Example 34. Preparations of purified pHisBotG protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotG protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotG protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotG antibodies to neutralize native C botulinum type G toxin is demonstrated using the mouse-C botulinum neutralization model described in Example 36.

EXAMPLE 50

Expression Of Recombinant Botulinal Toxin Proteins In Eucaryotic Host Cells

Recombinant botulinal C fragment proteins may be expressed in eucaryotic host cells, such as yeast and insect cells.

a) Expression In Yeast

5

10

15

20

25

30

Botulinal C fragments derived from serotypes A, B, C, D, E, F and G may be expressed in yeast cells using a variety of commercially available vectors. For example, the pPIC3K and pPIC9K expression vectors (Invitrogen) may be employed for expression in the methylotrophic yeast. *Pichia pastoris*. When the pPIC3K vector is employed, expression of the botulinal C fragment protein will be intracellular. When the pPIC3K vector is employed, the botulinal C fragment protein will be secreted (the alpha factor secretion signal is provided on the pPIC9K vector).

DNA sequences encoding the desired C fragment is inserted into these vectors using techniques known to the art. Briefly, the desired botulinal expression cassette (including sequences encoding the his-tag: described in the preceding examples) is amplified using the

PCR in conjunction with primers that incorporate unique restriction sites at the termini of the amplified fragment. Suitable restriction enzyme sites include *SnaBI*. *EcoRI*. *AvrII* and *NotI*. When the botulinal C fragment is to be expressed using the pPIC3K vector, the initiator methionine (ATG) is provided by the desired Bot gene sequence and a Kozak consensus sequence is engineered upstream of the ATG (e.g., ACCATGG).

The amplified restriction fragment containing the botulinal C fragment gene is then cloned into the desired expression vector. Recombinant clones are integrated into the *Pichia pastoris* genome and recombinant protein expression is induced using methanol following the manufacturer's instructions (Invitrogen Pichia expression kit manual).

C. hotulinum genes are A/T rich and contain multiple sequences that are similar to yeast transcriptional termination signals (e.g., TTTTTATA). If premature transcription termination is observed when the botulinal C fragment genes are expressed in yeast, the transcription termination signals present in the C fragment genes can be removed by either site directed mutagenesis (utilizing the pALTER system: Promega) or by construction of synthetic genes utilizing overlapping synthetic primers.

The botulinal C fragment genes may be expressed in other yeast cells using other commercially available vectors [e.g., using the pYES2 vector (Invitrogen) and S. cerevisiae cells (Invitrogen)].

b) Expression In Insect Cells

- 5

10

15

20

25

30

Botulinal C fragments derived from serotypes A. B. C. D. E. F and G may be expressed in insect cells using a variety of commercially available vectors. For example, the pBlueBac4 transfer vector (Invitrogen) may be employed for expression in *Spodopiera frugiperda* (Sf9) insect cells (baculovirus expression system) (equivalent baculovirus vectors and host cells are available from other vendors, e.g., Pharmingen, San Diego, CA). Botulinal C fragments contained on *Ncol/HindIII* fragments contained within the pHisBotA-G expression constructs (described in the preceding examples) are cloned into the pBlueBac4 vector (digested with *Ncol* and *HindIII*): the *Ncol* site present on the C fragment constructs overlaps with the start codon of the fusion proteins. In the case of botulinal C fragment clones that contain internal *HindIII* sites (e.g., using the BotG sequences described in Ex. 49), the C fragment gene is contained within a *Ncol/XhoI* fragment on the pHisBot construct. This *Ncol/XhoI* fragment is excised from pHisBot and inserted into pBlueBac4 digested with *Ncol* and *SalI*. Recombinant baculoviruses are made and the desired recombinant C fragment

is expressed in Sf9 cells using the protocols provided by the manufacturer (Invitrogen MaxBac manual). The resulting constructs will express the pHisBot protein intracellularly (including the N-terminal his-tag) under the control of the polyhedrin promoter. For extracellular secretion of botulinal C fragment proteins, the C fragment sequences from the pHisBot constructs are cloned into the pMelBacB vector (Invitrogen) as described above for the pBlueBac4 vector. When the pMelBacB vector is employed, the his-tagged botulinal C fragment proteins are secreted (utilizing a vector-encoded honeybee melittin secretion signal) and contain a nine amino acid extension at the N-terminus.

5

10

15

His-tagged botulinal C fragments expressed in yeast or insect cells are purified using metal chelation columns as described in the preceding examples.

From the above it is clear that the present invention provides compositions and methods for the preparation of effective multivalent vaccines against *C. hotulinum* neurotoxin. It is also contemplated that the recombinant botulinal proteins be used for the production of antitoxins. All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention.

SEQUENCE LISTING

```
(1) GENERAL INFORMATION:
   5
                (i) APPLICANT: Williams, James A.
                                Thalley, Bruce S.
              (ii) TITLE OF INVENTION: Multivalent Vaccine For Clostridium
                       Botulinum Neurotoxin
  10
             (iii) NUMBER OF SEQUENCES: 82
              (iv) CORRESPONDENCE ADDRESS:
                     (A) ADDRESSEE: Medlen & Carroll
  15
                     (B) STREET: 220 Montgomery Street, Suite 2200
                     (C) CITY: San Francisco
(D) STATE: California
                     (E) COUNTRY: United States of America
                     (F) ZIP: 94104
  20
               (v) COMPUTER READABLE FORM:
                    (A) MEDIUM TYPE: Floppy disk
                    (B) COMPUTER: IBM PC compatible (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 125
                    (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
              (vi) CURRENT APPLICATION DATA:
                    (A) APPLICATION NUMBER: US
                    (B) FILING DATE:
 30
                    (C) CLASSIFICATION:
           (viii) ATTORNEY/AGENT INFORMATION:
                    (A) NAME: Carroll, Peter G.
                    (B) REGISTRATION NUMBER: 32,837
 35
                    (C) REFERENCE/DOCKET NUMBER: OPHD-02959
             (ix) TELECOMMUNICATION INFORMATION:
                    (A) TELEPHONE: (415) 705-8410
(B) TELEFAX: (415) 397-8338
 40
        (2) INFORMATION FOR SEQ ID NO:1:
              (i) SEQUENCE CHARACTERISTICS:
                   (A) LENGTH: 24 base pairs
 45
                   (B) TYPE: nucleic acid
                   (C) STRANDEDNESS: single (D) TOPOLOGY: linear
             (ii) MOLECULE TYPE: DNA (genomic)
50
             (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
        GGAAATTTAG CTGCAGCATC TGAC
                                                                                       24
55
        (2) INFORMATION FOR SEQ ID NO:2:
              (i) SEQUENCE CHARACTERISTICS:
                   (A) LENGTH: 24 base pairs
                   (B) TYPE: nucleic acid
60
                   (C) STRANDEDNESS: single (D) TOPOLOGY: linear
            (ii) MOLECULE TYPE: DNA (genomic)
65
            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
       TCTAGCAAAT TCGCTTGTGT TGAA
                                                                                       24
       (2) INFORMATION FOR SEQ ID NO:3:
70
```

5	(i)	(B) TY (C) SI	CE CHARA ENGTH: 2 YPE: nuc TRANDEDN DPOLOGY:	0 base leic ac ESS: si	pairs id ngle								
	(ii)	MOLECUI	LE TYPE:	DNA (g	enomi	c)							
10	(xi)	SEQUENC	CE DESCR	IPTION:	SEQ	ID N	0:3:						
• • •	CTCGCATA	TA GCATT	TAGACC										20
	(2) INFO	RMATION	FOR SEQ	ID NO:	4:								
15	(i)	(B) TY (C) ST	ENGTH: 1: (PE: nuc. (RANDEDN)	9 base leic ac ESS: si	pairs id ngle								
20	1::5		OPOLOGY:										
		MOLECUL											
25	CTATCTAG	SEQUENC		IPTION:	SEQ	ID NO	D:4 :						
	(2) INFO			TD NO.	c .								19
		SEQUENC											
30	(1,	(A) LE (B) TY (C) ST	ENGTH: 8: PE: nuc: RANDEDNI POLOGY:	133 bas leic ac ESS: si	e pai id ngle	rs							
35	(ii)	MOLECUL	E TYPE:	DNA (g	enomi	c)							
	'(ix)	FEATURE			•								
40			ME/KEY: CATION:		0								
70	(xi)	SEQUENC	E DESCR	IPTION:	SEQ	ID NO	0:5:						
45	ATG TCT Met Ser	TTA ATA Leu Ile	TCT AAA Ser Lys 5	GAA GA Glu Gl	G TTA u Leu	ATA Ile 10	AAA Lys	CTC Leu	GCA Ala	TAT Tyr	AGC Ser 15	ATT Ile	48
50	AGA CCA A	AGA GAA Arg Glu 20	AAT GAG Asn Glu	TAT AA Tyr Ly	A ACT s Thr 25	ATA Ile	CTA Leu	ACT Thr	AAT Asn	TTA Leu 30	GAC Asp	GAA Glu	96
50	TAT AAT A	AAG TTA Lys Leu 35	ACT ACA Thr Thr	Asn As	T AAT n Asn 0	GAA Glu	AAT Asn	AAA Lys	TAT Tyr 45	TTG Leu	CAA Gln	TTA Leu	144
55	AAA AAA (Lys Lys) 50	CTA AAT Leu Asn	GAA TCA Glu Ser	ATT GA Ile As 55	T GTT p Val	TTT Phe	ATG Met	AAT Asn 60	AAA Lys	TAT Tyr	AAA Lys	ACT Thr	192
60	TCA AGC A Ser Ser A	AGA AAT Arg Asn	AGA GCA Arg Ala 70	CTC TC Leu Se	T AAT r Asn	CTA Leu	AAA Lys 75	AAA Lys	GAT Asp	ATA Ile	TTA Leu	AAA Lys 80	240
65	GAA GTA A	ATT CTT Ile Leu	ATT AAA Ile Lys 85	AAT TO Asn Se	C AAT r Asn	ACA Thr 90	AGC Ser	CCT Pro	GTA Val	GAA Glu	AAA Lys 95	AAT Asn	288
70	TTA CAT	TTT GTA Phe Val 100	TGG ATA Trp Ile	GGT GG Gly Gl	A GAA y Glu 105	GTC Val	AGT Ser	GAT Asp	ATT Ile	GCT Ala 110	CTT Leu	GAA Glu	336
70													

- 220 -

,	TAC	ATA Ile	Lys 115	GIn	TGG Trp	GCT Ala	GAT Asp	ATT Ile 120	Asn	GCA Ala	GAA Glu	TAT Tyr	AAT Asn 125	Ile	AAA Lys	CTG Leu		384
5	TGG Trp	TAT Tyr 130	Asp	AGT Ser	GAA Glu	GCA Ala	TTC Phe 135	TTA Leu	GTA Val	AAT Asn	ACA Thr	CTA Leu 140	Lys	AAG Lys	GCT Ala	ATA Ile		432
10	GTT Val 145	Glu	TCT	TCT Ser	ACC Thr	ACT Thr 150	GAA Glu	GCA Ala	TTA Leu	CAG Gln	CTA Leu 155	Leu	GAG Glu	GAA Glu	GAG Glu	ATT Ile 160		480
15	CAA Gln	AAT Asn	CCT Pro	CAA Gln	TTT Phe 165	GAT Asp	AAT Asn	ATG Met	AAA Lys	TTT Phe 170	Tyr	AAA Lys	AAA Lys	AGG Arg	ATG Met 175	GAA Glu		528
20	TTT Phe	ATA Ile	TAT Tyr	GAT Asp 180	AGA Arg	CAA Gln	AAA Lys	AGG Arg	TTT Phe 185	ATA Ile	AAT Asn	TAT Tyr	TAT Tyr	AAA Lys 190	TCT Ser	CAA Gln		576
,	ATC Ile	AAT Asn	AAA Lys 195	CCT Pro	ACA Thr	GTA Val	CCT Pro	ACA Thr 200	ATA Ile	GAT Asp	GAT Asp	ATT Ile	ATA Ile 205	AAG Lys	TCT Ser	CAT His		624
25	CTA Leu	GTA Val 210	TCT Ser	GAA Glu	TAT Tyr	AAT Asn	AGA Arg 215	GAT Asp	GAA Glu	ACT Thr	GTA Val	TTA Leu 220	GAA Glu	TCA Ser	TAT Tyr	AGA Arg		672
30	ACA Thr 225	AAT Asn	TCT Ser	TTG Leu	AGA Arg	AAA Lys 230	ATA Ile	AAT Asn	AGT Ser	AAT Asn	CAT His 235	GGG Gly	ATA Ile	GAT Asp	ATC Ile	AGG Arg 240		720
35	GCT Ala	AAT Asn	AGT Ser	TTG Leu	TTT Phe 245	ACA Thr	GAA Glu	CAA Gln	GAG Glu	TTA Leu 250	TTA Leu	AAT Asn	ATT Ile	TAT Tyr	AGT Ser 255	CAG Gln		768
40	GAG Glu	TTG Leu	TTA Leu	AAT Asn 260	CGT Arg	GGA Gly	AAT Asn	TTA Leu	GCT Ala 265	GCA Ala	GCA Ala	TCT Ser	GAC Asp	ATA Ile 270	GTA Val	AGA Arg		816
	TTA Leu	TTA Leu	GCC Ala 275	CTA Leu	AAA Lys	AAT Asn	TTT Phe	GGC Gly 280	GGA Gly	GTA Val	TAT Tyr	TTA Leu	GAT Asp 285	GTT Val	GAT Asp	ATG Met		864
45	CTT Leu	CCA Pro 290	GGT Gly	ATT Ile	CAC His	TCT Ser	GAT Asp 295	TTA Leu	TTT Phe	AAA Lys	ACA Thr	ATA Ile 300	TCT Ser	AGA Arg	CCT Pro	AGC Ser		912
50-	TCT Ser 305	ATT Ile	GGA Gly	CTA Leu	GAC Asp	CGT Arg 310	TGG Trp	Glu	ATG Met	Ile	Lys	Leu	GAG Glu	GCT Ala	Ile	ATG Met 320		960
55	AAG Lys	TAT Tyr	AAA Lys	AAA Lys	TAT Tyr 325	ATA Ile	AAT Asn	AAT Asn	TAT Tyr	ACA Thr 330	TCA Ser	GAA Glu	AAC Asn	TTT Phe	GAT Asp 335	AAA Lys		1008
60	CTT Leu	GAT Asp	CAA Gln	CAA Gln 340	TTA Leu	AAA Lys	GAT Asp	AAT Asn	TTT Phe 345	AAA Lys	CTC Leu	ATT Ile	ATA Ile	GAA Glu 350	AGT Ser	AAA Lys	;	1056
	AGT Ser	GAA Glu	AAA Lys 355	TCT Ser	GAG Glu	ATA Ile	TTT Phe	TCT Ser 360	AAA Lys	TTA Leu	GAA Glu	AAT Asn	TTA Leu 365	AAT Asn	GTA Val	TCT Ser	:	1104
65	GAT Asp	CTT Leu 370	GAA Glu	ATT Ile	AAA Lys	Ile	GCT Ala 375	TTC Phe	GCT Ala	TTA Leu	GGC Gly	AGT Ser 380	GTT Val	ATA Ile	AAT Asn	CAA Gln	;	1152

	GCC Ala 385	200	ATA Ile	TCA Ser	AAA Lys	CAA Gln 390	GGT Gly	TCA Ser	TAT	CTT Leu	ACT Thr 395	Asn	CTA Leu	GTA Val	ATA Ile	GAA Glu 400	1200
5	CAA Gln	GTA Val	AAA Lys	AAT Asn	AGA Arg 405	TAT Tyr	CAA Gln	TTT Phe	TTA Leu	AAC Asn 410	CAA Gln	CAC	CTT	AA C Asn	CCA Pro 415	GCC Ala	1248
10	ATA Ile	GAG Glu	TCT Ser	GAT Asp 420	AAT Asn	AAC Asn	TTC Phe	ACA Thr	GAT Asp 425	ACT Thr	ACT Thr	AAA Lys	ATT Ile	TTT Phe 430	CAT His	GAT Asp	1296
15	TCA Ser	TTA Leu	TTT Phe 435	AAT Asn	TCA Ser	GCT Ala	ACC Thr	GCA Ala 440	GAA Glu	AAC Asn	TCT Ser	ATG Met	TTT Phe 445	TTA Leu	ACA Thr	AAA Lys	1344
20	ATA Ile	GCA Ala 450	CCA Pro	TAC Tyr	TTA Leu	CAA Gln	GTA Val 455	GGT Gly	TTT Phe	ATG Met	CCA Pro	GAA Glu 460	GCT Ala	CGC Arg	TCC Ser	ACA Thr	1392
	ATA Ile 465	AGT Ser	TTA Leu	AGT Ser	GGT Gly	CCA Pro 470	GGA Gly	GCT Ala	TAT Tyr	GCG Ala	TCA Ser 475	GCT Ala	TAC Tyr	TAT Tyr	GAT Asp	TTC Phe 480	1440
25	ATA Ile	AAT Asn	TTA Leu	CAA Gln	GAA Glu 485	AAT Asn	ACT Thr	ATA Ile	GAA Glu	AAA Lys 490	ACT Thr	TTA Leu	AAA Lys	GCA Ala	TCA Ser 495	GAT Asp	1488
30	TTA Leu	ATA Ile	GAA Glu	TTT Phe 500	AAA Lys	TTC Phe	CCA Pro	GAA Glu	AAT Asn 505	AAT Asn	CTA Leu	TCT Ser	CAA Gln	TTG Leu 510	ACA Thr	GAA Glu	1536
35	CAA Gln	GAA Glu	ATA Ile 515	AAT Asn	AGT Ser	CTA Leu	TGG Trp	AGC Ser 520	TTT Phe	GAT Asp	CAA Gln	GCA Ala	AGT Ser 525	GCA Ala	AAA Lys	TAT Tyr	1584
40	CAA Gln	TTTT Phe 530	GAG Glu	AAA Lys	TAT Tyr	GTA Val	AGA Arg 535	GAT Asp	TAT Tyr	ACT Thr	GGT Gly	GGA Gly 540	TCT Ser	CTT Leu	TCT Ser	GAA Glu	1632
	GAC Asp 545	AAT Asn	GGG Gly	GTA Val	GAC Asp	TTT Phe 550	AAT Asn	AAA Lys	TAA Asn	ACT Thr	GCC Ala 555	CTC Leu	GAC Asp	AAA Lys	AAC Asn	TAT Tyr 560	1680
45	TTA Leu	TTA Leu	AAT Asn	AAT Asn	AAA Lys 565	ATT Ile	CCA Pro	TCA Ser	AAC Asn	AAT Asn 570	GTA Val	GAA Glu	GAA Glu	GCT Ala	GGA Gly 575	AGT Ser	1728
50	AAA Lys	AAT Asn	TAT Tyr	GTT Val 580	CAT His	TAT Tyr	ATC Ile	ATA Ile	CAG Gln 585	Leu	CAA Gln	GGA Gly	GAT Asp	GAT Asp 590	ATA Ile	AGT Ser	1776
55	TAT Tyr	GAA Glu	GCA Ala 595	ACA Thr	TGC Cys	AAT Asn	Leu	TTT Phe 600	TCT Ser	AAA Lys	AAT Asn	CCT Pro	AAA Lys 605	AAT Asn	AGT Ser	ATT Ile	1824
60	116	ATA Ile 610	CAA Gln	CGA Arg	AAT Asn	ATG Met	AAT Asn 615	GAA Glu	AGT Ser	GCA Ala	AAA Lys	AGC Ser 620	TAC Tyr	TTT Phe	TTA Leu	AGT Ser	1872
	GAT Asp 625	GAT Asp	GGA Gly	GAA Glu	TCT Ser	ATT Ile 630	TTA Leu	GAA Glu	TTA Leu	AAT Asn	AAA Lys 635	TAT Tyr	AGG Arg	ATA Ile	CCT Pro	GAA Glu 640 .	1920
65	AGA Arg	TTA Leu	AAA Lys	AAT Asn	AAG Lys 645	GAA Glu	AAA Lys	GTA Val	Lys	GTA Val 650	ACC Thr	TTT Phe	ATT Ile	GGA Gly	CAT His 655	GGT Gly	1968

	AAA Lys	GAT ASE	GAZ Glu	TTC Phe 660	: ASI	C ACA	AGC Ser	GAJ	A TTT	Ala	AGA Arg	TT!	A AG1	GT) Val	L As	TCA Ser		2016
5	CTI Leu	TCC Ser	AAT Asr 675	ı Gıu	ATA	AGT Ser	TCA Ser	TTT Phe 680	: Lei	GAT Asp	ACC Thr	ATA Ile	AAA Lys 685	Let	A GAT 1 Asp	T ATA		2064
10	261	690) Lys	ASI	vai	GIU	695	Asn	ı Lev	Leu	Gly	700	Asn	. Met	. Phe	AGT Ser		2112
15	705	Asp	Pne	ASN	vai	710	Glu	Thr	туг	Pro	715	Lys	Leu	Leu	Leu	AGT Ser 720		2160
20	116	Mec	Asp	Lys	725	inr	Ser	Thr	Leu	Pro 730	Asp	Val	Asn	Lys	735			2208
	116	1111	116	7 4 0	AIA	Asn	GIN	Tyr	G1u 745	Val	Arg	Ile	Asn	Ser 750	Glu	GGA Gly		2256
25	Arg	гλг	755	Leu	Leu	Ala	His	5er 760	Gly	Lys	Trp	Ile	Asn 765	Lys	Glu			2304
30	GCT Ala	ATT Ile 770	ATG Met	AGC Ser	GAT Asp	TTA	TCT Ser 775	AGT Ser	AAA Lys	GAA Glu	TAC Tyr	ATT Ile 780	TTT Phe	TTT Phe	GAT Asp	TCT Ser		2352
35	785	Asp	ASN	AAG Lys	Leu	790	Ala	Lys	Ser	Lys	Asn 795	Ile	Pro	Gly	Leu	Ala 800		2400
40	ser	ire	ser	GAA Glu	805	lie	Lys	Thr	Leu	Leu 810	Leu	Asp	Ala	Ser	Val 815	Ser		2448
	CCT Pro	GAT Asp	ACA Thr	AAA Lys 820	TTT Phe	ATT Ile	TTA Leu	AAT Asn	AAT Asn 825	CTT Leu	AAG Lys	CTT Leu	AAT Asn	ATT Ile 830	GAA Glu	TCT Ser	:	2496
45	TCT Ser	TTA 11e	GGG Gly 835	GAT Asp	TAC Tyr	ATT Ile	TAT Tyr	TAT Tyr 840	GAA Glu	AAA Lys	TTA Leu	GAG Glu	CCT Pro 845	GTT Val	AAA Lys	AAT Asn	:	2544
50	ATA Ile	ATT Ile 850	CAC His	AAT Asn	TCT Ser	ATA Ile	GAT Asp 855	GAT Asp	TTA Leu	ATA Ile	GAT Asp	GAG Glu 860	TTC Phe	AAT Asn	CTA Leu	CTT Leu	;	2592
55	GAA Glu 865	AAT Asn	GTA Val	TCT Ser	GAT Asp	GAA Glu 870	TTA Leu	TAT Tyr	GAA Glu	TTA Leu	AAA Lys 875	AAA Lys	TTA Leu	AAT Asn	AAT Asn	CTA Leu 880	2	2640
60	GAT Asp	GAG Glu	AAG Lys	TAT Tyr	TTA Leu 885	ATA Ile	TCT Ser	TTT Phe	GAA Glu	GAT Asp 890	ATC Ile	TCA Ser	AAA Lys	AAT Asn	AAT Asn 895	TCA Ser	2	2688
	ACT Thr	TAC Tyr	ser	GTA Val 900	AGA Arg	TTT Phe	ATT Ile	AAC Asn	AAA Lys 905	AGT Ser	AAT Asn	GGT Gly	GAG Glu	TCA Ser 910	GTT Val	TAT Tyr	2	2736
65	GTA Val	GIU	ACA Thr 915	GAA . Glu	AAA Lys	GAA . Glu	11e	TTT Phe 920	TCA Ser	AAA Lys	TAT Tyr	Ser	GAA Glu 925	CAT His	ATT Ile	ACA Thr	2	784

,	AAA Lys	GAA Glu 930	ATA Ile	AGT Ser	ACT Thr	ATA Ile	AAG Lys 935	AAT Asn	AGT Ser	ATA Ile	ATT Ile	ACA Thr 940	GAT Asp	GTT Val	AAT Asn	GGT Gly	2832
. 5	AAT Asn 945	TTA Leu	TTG Leu	GAT Asp	AAT Asn	ATA Ile 950	CAG Gln	TTA Leu	GAT Asp	CAT His	ACT Thr 955	TCT Ser	CAA Gln	GTT Val	AAT Asn	ACA Thr 960	2880
10	TTA Leu	AAC Asn	GCA Ala	GCA Ala	TTC Phe 965	TTT Phe	ATT Ile	CAA Gln	TCA Ser	TTA Leu 970	ATA Ile	GAT Asp	TAT Tyr	AGT Ser	AGC Ser 975	AAT Asn	2928
15	Lys	Asp	GTA Val	Leu 980	Asn	Asp	Leu	Ser	Thr 985	Ser	Val	Lys	Val	Gln 990	Leu	Tyr	2976
20	Ala	Gln	CTA Leu 995	Phe	Ser	Thr	Gly	Leu 1000	Asn)	Thr	Ile	Tyr	Asp 1009	Ser	Ile	Gln	3024
	TTA Leu	GTA Val 1010	AAT Asn)	TTA Leu	ATA Ile	TCA Ser	AAT Asn 1015	Ala	GTA Val	AAT Asn	GAT Asp	ACT Thr 1020	Ile	AAT Asn	GTA Val	CTA Leu	3072
25	CCT Pro 1029	Thr	ATA Ile	ACA Thr	GAG Glu	GGG Gly 1030	Ile	CCT Pro	ATT Ile	GTA Val	TCT Ser 1035	Thr	ATA Ile	TTA Leu	GAC Asp	GGA Gly 1040	3120
30	ATA Ile	AAC Asn	TTA Leu	GGT Gly	GCA Ala 1049	Ala	ATT Ile	AAG Lys	GAA Glu	TTA Leu 1050	Leu	GAC Asp	GAA Glu	CAT His	GAC Asp 1055	Pro	3168
35	TTA Leu	CTA Leu	AAA Lys	AAA Lys 1060	Glu	TTA Leu	GAA Glu	GCT Ala	AAG Lys 1069	Val	GGT Gly	GTT Val	TTA Leu	GCA Ala 1070	Ile	AAT Asn	3216
40	ATG Met	TCA Ser	TTA Leu 1075	Ser	ATA Ile	GCT Ala	GCA Ala	ACT Thr 1080	Val	GCT Ala	TCA Ser	ATT Ile	GTT Val 1085	Gly	ATA Ile	GGT Gly	3264
	GCT Ala	GAA Glu 1090	GTT Val)	ACT Thr	ATT Ile	TTC Phe	TTA Leu 1095	Leu	CCT Pro	ATA Ile	GCT Ala	GGT Gly 1100	Ile	TCT Ser	GCA Ala	GGA Gly	3312
45	ATA Ile 1109	Pro	TCA Ser	TTA Leu	GTT Val	AAT Asn 1110	Asn	GAA Glu	TTA Leu	ATA Ile	TTG Leu 1115	His	GAT Asp	AAG Lys	GCA Ala	ACT Thr 1120	3360
50	TCA Ser	GTG Val	GTA Val	AAC Asn	TAT Tyr 1125	Phe	AAT Asn	CAT His	TTG Leu	TCT Ser 1130	Glu	TCT Ser	AAA Lys	AAA Lys	TAT Tyr 1135	Gly	3408
55	CCT Pro	CTT Leu	AAA Lys	ACA Thr 1140	Glu	GAT Asp	GAT Asp	AAA Lys	ATT Ile 1145	Leu	GTT Val	CCT Pro	ATT Ile	GAT Asp 1150	Asp	TTA Leu	3456
60	GTA Val	ATA Ile	TCA Ser 115	Glu	ATA Ile	GAT Asp	TTT Phe	AAT Asn 1160	Asn	AAT Asn	TCG Ser	ATA Ile	AAA Lys 1169	Leu	GGA Gly	ACA Thr	3504
	TGT Cys	AAT Asn 1170	ATA Ile	TTA Leu	GCA Ala	ATG Met	GAG Glu 1175	Gly	GGA Gly	TCA Ser	GGA Gly	CAC His 1180	Thr	GTG Val	ACT Thr	GGT Gly	3552
65	AAT Asn 118	Ile	GAT Asp	CAC His	TTT Phe	TTC Phe 1190	Ser	TCT Ser	CCA Pro	TCT Ser	ATA Ile 1195	Ser	TCT Ser	CAT His	ATT Ile	CCT Pro 1200	3600

•	TCA Ser	TTA Leu	TCA Ser	ATT Ile	TAT Tyr 120	Ser	GCA Ala	ATA Ile	GGT Gly	ATA Ile 121	Glu	ACA Thr	GAA Glu	AA] Asr	CTA Leu 121	GAT Asp	3648
5	TTT Phe	TCA Ser	AAA Lys	AAA Lys 122	TTe	ATG Met	ATG Met	TTA Leu	CCT Pro 122	Asn	GCT Ala	CCT Pro	TCA Ser	AGA Arg	Val	TTT Phe	3696
10	TGG Trp	TGG Trp	GAA Glu 123	THE	GGA Gly	GCA Ala	GTT Val	CCA Pro 124	GIY	TTA Leu	AGA Arg	TCA Ser	TTG Leu 124	Glu	AAT Asn	GAC Asp	3744
15	GGA Gly	ACT Thr 125	Arg	TTA Leu	CTT Leu	GAT Asp	TCA Ser 125	Ile	AGA Arg	GAT Asp	TTA Leu	TAC Tyr 126	Pro	GGT Gly	AAA Lys	TTT Phe	3792
20	TAC Tyr 1265	ıгр	AGA Arg	TTC Phe	TAT Tyr	GCT Ala 127	Phe	TTC Phe	GAT Asp	TAT Tyr	GCA Ala 127	Ile	ACT Thr	ACA Thr	TTA Leu	AAA Lys 1280	3840
	CCA Pro	GTT Val	TAT Tyr	GAA Glu	GAC Asp 128	Thr	AAT Asn	ATT Ile	AAA Lys	ATT Ile 1290	Lys	CTA Leu	GAT Asp	AAA Lys	GAT Asp 129		3888
25	AGA Arg	AAC Asn	TTC Phe	ATA Ile 1300	мес	CCA Pro	ACT Thr	ATA Ile	ACT Thr 130	Thr	AAC Asn	GAA Glu	ATT Ile	AGA Arg 131	Asn	AAA Lys	3936
30	Leu	ser	1315	ser	Phe	GAT Asp	GIY	Ala 1320	Gly	Gly	Thr	Tyr	Ser 1329	Leu	Leu	Leu	3984
35	TCT Ser	TCA Ser 1330	ıyr	CCA Pro	ATA Ile	TCA Ser	ACG Thr 1335	Asn	ATA Ile	AAT Asn	TTA Leu	TCT Ser 1340	Lys	GAT Asp	GAT Asp	TTA Leu	4032
40	TGG Trp 1345	TIE	TTT Phe	AAT Asn	ATT Ile	GAT Asp 1350	Asn	GAA Glu	GTA Val	AGA Arg	GAA Glu 1355	Ile	TCT Ser	ATA Ile	GAA Glu	AAT Asn 1360	4080
	GGT Gly	ACT Thr	ATT Ile	AAA Lys	AAA Lys 1365	GIÀ	AAG Lys	TTA Leu	ATA Ile	AAA Lys 1370	Asp	GTT Val	TTA Leu	AGT Ser	AAA Lys 1379	Ile	4128
45	GAT . Asp	ATA Ile	ASII	AAA Lys 1380	ASD	AAA Lys	CTT Leu	ATT Ile	ATA Ile 1385	Gly	AAT Asn	CAA Gln	ACA Thr	ATA Ile 1390	Asp	TTT Phe	4176
50	TCA Ser	GIA	GAT Asp 1395	TTG	GAT Asp	AAT Asn	Lys	GAT Asp 1400	Arg	Tyr	Ile	Phe	TTG Leu 1405	Thr	тст Суз	GAG Glu	4224
55	TTA (GAT Asp 1410	Asp .	AAA Lys	ATT Ile	ser	TTA Leu 1415	lie	ATA Ile	GAA . Glu	Ile .	AAT Asn 1420	Leu	GTT Val	GCA Ala	AAA Lys	4272
60	TCT Ser 1425	rat . ryr :	AGT ' Ser :	TTG ' Leu :	Leu	TTG Leu 1430	TCT	GGG Gly	GAT Asp	Lys .	AAT Asn 1435	Tyr	TTG Leu	ATA Ile	TCC Ser	AAT Asn 1440	4320
	TTA 1	TCT A	AAT A	unr .	ATT Ile 1445	GAG (Glu)	AAA . Lys	ATC Ile	Asn	ACT Thr 1	TTA (Leu (GGC Gly	CTA (Leu ,	Asp	AGT Ser 1455	Lys	4368

	AAT Asn	ATA Ile	GCG Ala	TAC Tyr 146	Asn	TAC Tyr	ACT Thr	GAT Asp	GAA Glu 146	Ser	AAT Asn	AAT Asn	AAA Lys	TAT Tyr 1470	Phe	GGA Gly	4416
5	GCT Ala	ATA Ile	TCT Ser 147	Lys	ACA Thr	AGT Ser	CAA Gln	AAA Lys 148	Ser	ATA Ile	ATA Ile	CAT His	TAT Tyr 148	Lys	AAA Lys	GAC Asp	4464
10	AGT Ser	AAA Lys 1490	Asn	ATA Ile	TTA Leu	GAA Glu	TTT Phe 1495	Tyr	AAT Asn	GAC Asp	AGT Ser	ACA Thr 1500	TTA Leu)	GAA Glu	TTT Phe	AAC Asn	4512
15	AGT Ser 1505	Lys	GAT Asp	TTT Phe	ATT Ile	GCT Ala 1510	Glu	GAT Asp	λTA Ile	AAT Asn	GTA Val 1519	Phe	ATG Met	AAA Lys	GAT Asp	GAT Asp 1520	4560
20	ATT Ile	AAT Asn	ACT Thr	ATA Ile	ACA Thr 1529	Gly	AAA Lys	TAC Tyr	TAT Tyr	GTT Val 1530	λsp	AAT Asn	AAT Asn	ACT Thr	GAT Asp 1535	Lys	4608
	AGT Ser	ATA Ile	GAT Asp	TTC Phe 1540	Ser	ATT Ile	TCT Ser	TTA Leu	GTT Val 1545	Ser	AAA Lys	AAT Asn	CAA Gln	GTA Val 1550	Lys	GTA Val	4656
25	AAT Asn	GGA Gly	TTA Leu 1555	Tyr	TTA Leu	AAT Asn	GAA Glu	TCC Ser 1560	Val	TAC Tyr	TCA Ser	TCT Ser	TAC Tyr 1565	Leu	GAT Asp	TTT Phe	4704
30	GTG Val	AAA L ys 1570	Asn	TCA Ser	GAT Asp	GGA Gly	CAC His 1579	His	AAT Asn	ACT Thr	TCT Ser	AAT Asn 1580	TTT Phe	ATG Met	AAT Asn	TTA Leu	4752
35	TTT Phe 1589	reu	GAC Asp	AAT Asn	ATA Ile	AGT Ser 1590	Phe	TGG Trp	AAA Lys	TTG Leu	TTT Phe 1595	Gly	TTT Phe	GAA Glu	AAT Asn	ATA Ile 1600	4800
40	AAT Asn	TTT Phe	GTA Val	ATC Ile	GAT Asp 1605	Lys	TAC Tyr	TTT Phe	ACC Thr	CTT Leu 1610	Val	GGT Gly	AAA Lys	ACT Thr	AAT Asn 1619	Leu	4848
•••	GGA Gly	TAT Tyr	GTA Val	GAA Glu 1620	Phe	ATT Ile	TG T Cys	GAC Asp	AAT Asn 1625	Asn	AAA Lys	AAT Asn	ATA Ile	GAT Asp 1630	Ile	TA T Tyr	4896
45	TTT Phe	GGT Gly	GAA Glu 1635	Trp	AAA Lys	ACA Thr	TCG Ser	TCA Ser 1640	Ser	AAA Lys	AGC Ser	ACT Thr	ATA Ile 1645	Phe	AGC Ser	GGA Gly	4944
50	TAA naA	GGT Gly 1650	Arg	AAT Asn	GTT Val	GTA Val	GTA Val 1655	Glu	CCT Pro	ATA Ile	TAT Tyr	AAT Asn 1660	CCT Pro	GAT Asp	ACG Thr	GGT Gly	4992
55	GAA Glu 1665	Asp	ATA Ile	TCT Ser	ACT Thr	TCA Ser 1670	Leu	GAT Asp	TT T Phe	TCC Ser	TAT Tyr 1679	Glu	CCT Pro	CTC Leu	TAT Tyr	GGA Gly 1680	5040
60	ATA Ile	GAT Asp	AGA Arg	TAT Tyr	ATA Ile 1689	Asn	AAA Lys	GTA Val	TTG Leu	ATA Ile 1690	Ala	CCT Pro	GAT Asp	TTA Leu	TAT Tyr 1695	Thr	5088
70	AGT Ser	TTA Leu	ATA Ile	AAT Asn 1700	Ile	TAA neA	ACC Thr	AAT Asn	TAT Tyr 1705	Tyr	TCA Ser	AAT Asn	GAG Glu	ΤΛC Τyr 1710	Tyr	CCT Pro	5136
65	GAG Glu	ATT	ATA Ile 1715	Val	CTT Leu	AAC Asn	CCA Pro	AAT Asn 1720	Thr	TTC Phe	CAC His	AAA Lys	AAA Lys 1725	Val	AAT Asn	ATA Ile	5184

	AAT Asn	TTA Leu 173	. wet	C AGT	TCT Ser	TCT Ser	TTT Phe 173	GIU	TAT Tyr	AAA Lys	TGG Trp	TCT Ser 174	Thi	A GAI	A GGA	A AGT / Ser	5232
5	GAC Asp 174	FILE	ATT Ile	TTA Leu	GTI Val	AGA Arg 175	Tyr	TTA Leu	GAA Glu	GAA Glu	AGT Ser 175	Asn	AAA Lys	AAA Lys	ATA Ile	TTA Leu 1760	5280
10	J.,	Lys	116	HIG	176	Lys 5	GIY	11e	Leu	177	Asn O	Thr	Gln	Ser	Phe 177		5328
15	275	Het	Sel	178	0	Pne	rys	Asp	11e 178	Lys 5	AAA Lys	Leu	Ser	Leu 179	Gly 0	Tyr	5376
20		Hec	179	5	Pne	Lys	ser	180	Asn 0	Ser	GAA Glu	Asn	Glu 180	Leu 5	Asp	Arg	5424
	Asp	His 181	Leu	GGA Gly	TTT Phe	AAA Lys	ATA Ile 1819	ile	GAT Asp	AAT Asn	AAA Lys	ACT Thr 182	Tyr	TAC Tyr	TAT Tyr	GAT Asp	5472
25	GAA Glu 1825	~3p	AGT Ser	AAA Lys	TTA Leu	GTT Val 1830	Lys	GGA Gly	TTA Leu	ATC Ile	AAT Asn 1835	Ile	AAT Asn	AAT Asn	TCA Ser	TTA Leu 1840	5520
30	TTC Phe	TAT Tyr	TTT Phe	GAT Asp	CCT Pro 1845	TTE	GAA Glu	TTT Phe	AAC Asn	TTA Leu 1850	GTA Val	ACT Thr	GGA Gly	TGG Trp	CAA Gln 185	Thr	5568
35		A311	Gly	1860	D	ryr	Tyr	Pne	1865	Ile	AAT Asn	Thr	Gly	Ala 1870	Ala)	Leu	5616
40	****	261	1875	Lys	iie	11e	Asn	1880	Lys	His	TTT Phe	Tyr	Phe 1885	Asn	Asn	Asp	5664
	Oly.	1890)	GIII	Leu	GIY	vai 1895	Pne	Lys	Gly		Asp 1900	Gly)	Phe	Glu	Tyr	5712
45	1905	Ala	PIO	MIA	ASI	1910	GIn	Asn	Asn	Asn	ATA Ile 1915	Glu	Gly	Gln	Ala	lle 1920	5760
50	GTT Val	1 7 1	GTII	ser	1925	Pne	Leu	Thr	Leu	Asn 1930	Gly	Lys	Lys	Tyr	Tyr 1935	Phe	5808
55	GAT Asp	1311	ASII	1940	Lys	Ala	vai	inr	G1y 1945	Trp	Arg	Ile	Ile	Asn 1950	Asn	Glu	5856
60	AAA ' Lys '	. y .	1955	FIIE	ASH	PIO 1	ASN A	1960	Ala	IIe	Ala A	Ala	Va l 1965	Gly	Leu	Gln	5904
	GTA /	ATT Ile 1970	GAC Asp	AAT Asn	AAT . Asn .	Lys :	TAT 1 Tyr 1 1975	FAT '	TTC . Phe .	AAT Asn	Pro A	GAC Asp 1980	ACT Thr	GCT Ala	ATC . Ile	ATC Ile	5952
65	TCA A Ser I 1985	AAA (GGT '	TGG (GIN	ACT (Thr \ 1990	STT /	AAT (Asn (GGT A	Ser .	AGA 1 Arg 1	rac ' Tyr '	TAC Tyr	TTT (Phe	Asp '	ACT Thr 2000	6000

	GAT Asp	ACC Thr	GCT Ala	ATT Ile	GCC Ala 200	Phe	AAT Asn	GGT Gly	TAT Tyr	AAA Lys 201	Thr	ATT Ile	GAT Asp	GGT Gly	AAA Lys 201	His	6048
5	TTT Phe	TAT Tyr	TTT Phe	GAT Asp 2020	Ser	GAT Asp	TGT Cys	GTA Val	GTG Val 2029	Lys	ATA Ile	GGT Gly	GTG Val	TTT Phe 203	Ser	ACC Thr	6096
10	TCT Ser	AAT Asn	GGA Gly 203	Phe	GAA Glu	TAT Tyr	TTT Phe	GCA Ala 2040	Pro	GCT Ala	AAT Asn	ACT Thr	TAT Tyr 204	Asn	AAT Asn	AAC Asn	6144
15	ATA Ile	GAA Glu 2050	GΙΆ	CAG Gln	GCT Ala	ATA Ile	GTT Val 2055	Tyr	CAA Gln	AGT Ser	AAA Lys	TTC Phe 2060	Leu	ACT Thr	TTG Leu	AAT Asn	6192
20	GGT Gly 2069	AAA Lys	AAA Lys	TAT Tyr	TAC Tyr	TTT Phe 2070	Asp	AAT Asn	AAC Asn	TCA Ser	AAA Lys 2079	Ala	GTT Val	ACC Thr	GGA Gly	TTG Leu 2080	6240
	CAA Gln	ACT Thr	ATT Ile	GAT Asp	AGT Ser 2089	Lys	AAA Lys	TAT Tyr	TAC Tyr	TTT Phe 2090	Asn	ACT Thr	AAC Asn	ACT Thr	GCT Ala 2099	Glu	6288
25'	GCA Ala	GCT Ala	ACT Thr	GGA Gly 2100	Trp	CAA Gln	ACT Thr	ATT Ile	GAT Asp 2109	Gly	AAA Lys	AAA Lys	TAT Tyr	TAC Tyr 2110	Phe	AAT Asn	6336
30	ACT Thr	AAC Asn	ACT Thr 2119	Ala	GAA Glu	GCA Ala	GCT Ala	ACT Thr 2120	Gly	TGG Trp	CAA Gln	ACT Thr	ATT Ile 2129	Asp	GGT Gly	AAA Lys	6384
35	AAA Lys	TAT Tyr 2130	Tyr	TTT Phe	AAT Asn	ACT Thr	AAC Asn 2135	Thr	GCT Ala	ATA Ile	GCT Ala	TCA Ser 2140	Thr	GGT Gly	TAT Tyr	ACA Thr	6432
40	ATT Ile 2145	ATT lle	AAT Asn	GGT Gly	AAA Lys	CAT His 2150	Phe	TAT Tyr	TTT Phe	AAT Asn	ACT Thr 2155	Asp	GGT Gly	ATT Ile	ATG Met	CAG Gln 2160	6480
	ATA Ile	GGA Gly	GTG Val	TTT Phe	AAA Lys 2169	Gly	CCT Pro	AAT Asn	GGA Gly	TTT Phe 2170	Glu	TAT Tyr	TTT Phe	GCA Ala	CCT Pro 2179	Ala	6528
45	AAT Asn	ACG Thr	GAT Asp	GCT Ala 2180	Asn	AAC Asn	ATA Ile	GAA Glu	GGT Gly 2185	Gln	GCT Ala	ATA Ile	CTT Leu	TAC Tyr 2190	Gln	AAT Asn	6576
50	GAA Glu	TTC Phe	TTA Leu 2199	Thr	TTG Leu	AAT Asn	GGT Gly	AAA Lys 2200	Lys	TAT Tyr	TAC Tyr	TTT Phe	GGT Gly 2209	Ser	GAC Asp	TCA Ser	6624
55	AAA Lys	GCA Ala 2210	Val	ACT Thr	GGA Gly	TGG Trp	AGA Arg 2215	Ile	ATT Ile	AAC Asn	AAT Asn	AAG Lys 2220	Lys	TAT Tyr	TAC Tyr	TTT Phe	6672
60	AAT Asn 2229	CCT Pro	AAT Asn	AAT Asn	GCT Ala	ATT 11e 2230	Ala	GCA Ala	ATT Ile	CAT His	CTA Leu 2235	Cys	ACT Thr	ATA Ile	AAT Asn	AAT Asn 2240	6720
	GAC Asp	AAG Lys	TAT Tyr	TAC Tyr	TTT Phe 2245	Ser	TAT Tyr	GAT Asp	GGA Gly	ATT Ile 2250	Leu	CAA Gln	AAT Asn	GGA Gly	TAT Tyr 2259	Ile	6768
65	ACT Thr	ATT Ile	GAA Glu	AGA Arg 2260	Asn	AAT Asn	TTC Phe	TAT Tyr	TTT Phe 226	Asp	GCT Ala	AAT Asn	AAT Asn	GAA Glu 2270	Ser	AAA Lys	6816

	ATG Met	GTA Val	ACA Thr 227	GTA	GTA Val	TTI Phe	AAA Lys	GGA Gly 228	Pro	AAT Asn	GG#	TTT Phe	GAG Glu 228	Tyr	TTT Phe	GCA Ala	6864
5	CCT Pro	GCT Ala 229	ASD	ACT Thr	CAC His	AAT Asn	AAT Asn 229	Asn	ATA Ile	GAA Glu	GGT Gly	CAG Gln 230	Ala	ATA Ile	GTT Val	TAC Tyr	6912
10	CAG Gln 230	ASII	AAA Lys	TTC Phe	TTA Leu	ACT Thr 231	Leu	AAT Asn	GGC Gly	AAA Lys	AAA Lys 231	Tyr	TAT Tyr	TTT Phe	GAT Asp	AAT Asn 2320	6960
15	GAC Asp	TCA Ser	AAA Lys	GCA Ala	GTT Val 232	Thr	GGA Gly	TGG Trp	CAA Gln	ACC Thr 233	Ile	GAT Asp	GGT Gly	AAA Lys	AAA Lys 233	Tyr	7008
20	. 7 .	rne	ASII	234		Thr	Ala	GIu	A1a 234	Ala 5	Thr	Gly	Trp	Gln 235	Thr O	Ile	7056
	GAT Asp	GGT Gly	AAA Lys 235	ьys	TAT Tyr	TAC Tyr	TTT Phe	AAT Asn 236	Leu	AAC Asn	ACT Thr	GCT Ala	GAA Glu 236	Ala	GCT Ala	ACT Thr	7104
25	GGA Gly	TGG Trp 2370	Gin	ACT Thr	ATT Ile	GAT Asp	GGT Gly 2375	Lys	AAA Lys	TAT Tyr	TAC Tyr	TTT Phe 238	Asn	ACT Thr	AAC Asn	ACT Thr	7152
30	TTC Phe 2389	Tie	GCC Ala	TCA Ser	ACT Thr	GGT Gly 2390	Tyr	ACA Thr	AGT Ser	ATT Ile	AAT Asn 239	Gly	AAA Lys	CAT His	TTT Phe	TAT Tyr 2400	7200
35	TTT Phe	AAT Asn	ACT Thr	GAT Asp	GGT Gly 2405	He	ATG Met	CAG Gln	ATA Ile	GGA Gly 241	Val	TTT Phe	AAA Lys	GGA Gly	CCT Pro 2415	Asn	7248
40	GGA Gly	TTT Phe	GAA Glu	TAC Tyr 2420	TTT Phe	GCA Ala	CCT Pro	GCT Ala	AAT Asn 2425	Thr	GAT Asp	GCT Ala	AAC Asn	AAC Asn 2430	Ile	GAA Glu	7296
	GG T Gly	CAA Gln	GCT Ala 2435	He	CTT Leu	TAC Tyr	CAA Gln	AAT Asn 2440	Lys	TTC Phe	TTA Leu	ACT Thr	TTG Leu 2445	Asn	GGT Gly	AAA Lys	7344
45	AAA Lys	TAT Tyr 2450	Tyr	TTT Phe	GGT Gly	AGT Ser	GAC Asp 2455	Ser	AAA Lys	GCA Ala	GTT Val	ACC Thr 2460	Gly	CTG Leu	CGA Arg	ACT Thr	7392
50	ATT Ile 2465	ASP	GGT Gly	AAA Lys	AAA Lys	TAT Tyr 2470	Tyr	Phe	AAT Asn	Thr	Asn	Thr	GCT Ala	GTT Val	Ala	GTT Val 2480	7440
55	ACT Thr	GGA Gly	TGG Trp	CAA Gln	ACT Thr 2485	Ile	AAT Asn	GGT Gly	Lys	AAA Lys 2490	Tyr	TAC Tyr	TTT Phe	AAT Asn	ACT Thr 2495	Asn	7488
60	ACT Thr	TCT Ser	ATA Ile	GCT Ala 2500	Ser	ACT Thr	GGT Gly	Tyr	ACA Thr 2505	Ile	ATT Ile	AGT Ser	Gly	AA A Lys 2510	His	TT T Phe	7536
	TAT Tyr	Pne .	AAT Asn 2515	Thr	GAT Asp	GGT Gly	Ile	ATG Met 2520	CAG . Gln	ATA Ile	GGA Gly	Val	TTT Phe 2525	AAA Lys	GGA Gly	ССТ Pro	7584
65	GAT (GGA Gly 2530	TTT Phe	GAA Glu	TAC Tyr	Pne	GCA Ala 2535	CCT Pro .	GCT . Ala .	AAT Asn	Thr	GAT Asp 2540	Ala .	AAC Asn	AAT . Asn	ATA Ile	7632

	GAA Glu 254	GIA	CAA Gln	GCT Ala	ATA Ile	CGT Arg 2550	Tyr	CAA Gln	AAT Asn	AGA Arg	TTC Phe 255	Leu	TAT Tyr	TTA Leu	CAT His	GAC Asp 2560	7680
. 5	AAT Asn	ATA Ile	TAT Tyr	TAT Tyr	TTT Phe 2569	GIY	AAT Asn	AAT Asn	TCA Ser	AAA Lys 2570	Ala	GCT Ala	ACT Thr	GGT Gly	TGG Trp 2579	Val	7728
10	ACT Thr	ATT Ile	GAT Asp	GGT Gly 2580	Asn	AGA Arg	TAT Tyr	TAC Tyr	TTC Phe 2585	Glu	CCT Pro	AAT Asn	ACA Thr	GCT Ala 2590	ATG Met O	GGT Gly	7776
15	GCG Ala	AAT Asn	GGT Gly 2595	Tyr	AAA Lys	ACT Thr	ATT Ile	GAT Asp 2600	Asn	AAA Lys	AAT Asn	TTT Phe	TAC Tyr 2609	Phe	AGA Arg	AAT Asn	7824
20	GIY	2610)	GIn	He	GIY	Val 2619	Phe	Lys	Gly	Ser	Asn 2620	Gly	Phe	GAA Glu	Tyr	7872
	TTT Phe 2625	Ата	CCT Pro	GCT Ala	AAT Asn	ACG Thr 2630	Asp	GCT Ala	AAC Asn	AAT Asn	ATA Ile 2639	Glu	GGT Gly	CAA Gln	GCT Ala	ATA Ile 2640	7920
25	CGT Arg	TAT Tyr	CAA Gln	TAA Asn	AGA Arg 2645	Phe	CTA Leu	CAT His	TTA Leu	CTT Leu 2650	Gly	AAA Lys	ATA Ile	TAT Tyr	TAC Tyr 2655	Phe	7968
30	GGT Gly	AAT Asn	AAT Asn	TCA Ser 2660	Lys	GCA Ala	GTT Val	ACT Thr	GGA Gly 2665	Trp	CAA Gln	ACT Thr	ATT Ile	AAT Asn 2670	GGT Gly	AAA Lys	8016
35	GTA Val	TAT Tyr	TAC Tyr 2675	Phe	ATG Met	CCT Pro	GAT Asp	ACT Thr 2680	Ala	ATG Met	GCT Ala	GCA Ala	GCT Ala 2685	Gly	GGA Gly	CTT Leu	8064
40	TTC Phe	GAG Glu 2690	He	GAT Asp	GGT Gly	GTT Val	ATA Ile 2695	Tyr	TTC Phe	TTT Phe	GGT Gly	GTT Val 2700	Asp	GGA Gly	GTA Val	AAA Lys	8112
	GCC Ala 2705	Pro	GGG Gly	ATA Ile	TAT Tyr	GGC Gly 2710											8133
45	(2)	INFO	RMAT	NOI	FOR	SEQ	ID N	10 : 6 :									
50		((i) S	(A) (B)	LEN TYP	CHAR IGTH: PE: a POLOG	271 mino	.0 ап о асі	nno d	acid	ls						
		(1	.i) M	OLEC	ULE	TYPE	: pr	otei	.n								
55						DESC											
	Met 1	Ser	Leu	Ile	Ser 5	Lys	Glu	Glu	Leu	Ile 10	Lys	Leu	Ala	Туr	Ser 15	Ile	
60	Λrg	Pro	Arg	Glu 20	Asn	Glu	Tyr	Lys	Thr 25	Ile	Leu	Thr	Asn	Leu 30	Asp	Glu	
	Tyr	Asn	Lys 35	Leu	Thr	Thr	Asn	Asn 40	Asn	Glu	Asn	Lys	Tyr 45	Leu	Gln	Leu	
65	Lys	Lys 50	Leu	Asn	Glu	Ser	Ile 55	Asp	Val	Phe	Met	Asn 60	Lys	Tyr	Lys	Thr	
70	Ser 65	Ser	Arg	Asn	Arg	Ala 70	Leu	Ser	Asn	Leu	Lys 75	Lys	Asp	Ile	Leu	Lys 80	

	Glu	Val	Ile	Leu	Ile 85	Lys	Asn	Ser	Asn	Thr 90		Pro	Val	Glu	Lys 95	Asn
5	Leu	His	Phe	Val 100	Trp	Ile	Gly	Gly	Glu 105		Ser	Asp	Ile	Ala 110		Glu
	Tyr	Ile	Lys 115	Gln	Trp	Ala	Asp	Ile 120	Asn	Ala	Glu	Tyr	Asn 125		Lys	Leu
10	Trp	Tyr 130	Asp	Ser	Glu	Ala	Phe 135	Leu	Val	Asn	Thr	Leu 140		Lys	Ala	Ile
15	Val 145	Glu	Ser	Ser	Thr	Thr 150	Glu	Ala	Leu	Gln	Leu 155	Leu	Glu	Glu	Glu	Ile 160
	Gln	Asn	Pro	Gln	Phe 165	Asp	Asn	Met	Lys	Phe 170		Lys	Lys	Arg	Met 175	
20	Phe	Ile	Tyr	Asp 180	Arg	Gln	Lys	Arg	Phe 185	Ile	Asn	Tyr	Tyr	Lys 190	Ser	Gln
	Ile	Asn	Lys 195	Pro	Thr	Val	Pro	Thr 200	Ile	Asp	Asp	Ile	Ile 205	Lys	Ser	His
25	Leu	Val 210	Ser	Glu	Tyr	Asn	Arg 215	Asp	Glu	Thr	Val	Leu 220	Glu	Ser	Tyr	Arg
30	Thr 225	Asn	Ser	Leu	Arg	Lys 230	Ile	Asn	Ser	Asn	His 235	Gly	Ile	Asp	Ile	Arg 240
	Ala	Asn	Ser	Leu	Phe 245	Thr	Glu	Gln	Glu	Leu 250	Leu	Asn	Ile	Tyr	Ser 255	Gln
35				Asn 260					265					270		-
•			2/5	Leu				280					285			
40		290		Ile			295					300				
45	305			Leu		310					315					320
				Lys	325					330					335	-
50				Gln 340					345					350		-
2.5			355	Ser				360					365			
55		370		Ile			375					380				
60	385			Ser		390					395					400
					405					410					415	
65				Asp 420					425					430		-
	Ser	Leu	Phe 435	Asn	Ser	Ala	Thr	Ala 440	Glu	Asn	Ser	Met	Phe 445	Leu	Thr	Lys

	Ile	Ala 450	Pro	Tyr	Leu	Gln	Val 455	Gly	Phe	Met	Pro	Glu 460	Ala	Arg	Ser	Thr
5	Ile 465	Ser	Leu	Ser	Gly	Pro 470	Gly	Ala	Tyr	Ala	Ser 475	Ala	Tyr	Tyr	Asp	Phe 480
	Ile	Asn	Leu	Gln	Glu 485	Asn	Thr	Ile	Glu	Lys 490	Thr	Leu	Lys	Ala	Ser 495	Asp
10	Leu	Ile	Glu	Phe 500	Lys	Phe	Pro	Glu	Asn 505	Asn	Leu	Ser	Gln	Leu 510		Glu
15			Ile 515					520					525			
		250	Glu				535					540				
20	3.3		Gly			350					555					560
25			Asn		303					570					575	
25			Tyr	300					585					590		
30			Ala 595					600					605			
		010	Gln				615					620				
35	323		Gly			630					635					640
40			Lys		045					650					655	
40			Glu	660					665					670		
45			Asn 675					680					685			
		0.70	Lys				635					700				
50	, 0.5		Phe			110					715					720
55			Asp		125					730					735	
			Ile	740					745					750		
60			Glu 755					760					765			
		, , ,	Met				775					780				
65	,03		Asn			790					795					800
	261	116	Ser	GIU	805	116	Lys	Thr	Leu	Leu 810	Leu	Asp	Ala	Ser	Val 815	Ser

	Pro	Asp	Thr	Lys 820	Phe	Ile	Leu	Asn	Asn 825	Leu	Lys	Leu	Asn	Ile 830	Glu	Ser
5	Ser	Ile	Gly 835	Asp	Tyr	Ile	туг	Tyr 840	Glu	Lys	Leu	Glu	Pro 845		Lys	Asn
	Ile	Ile 850	His	Asn	Ser	Ile	Asp 855	Asp	Leu	Ile	Asp	Glu 860	Phe	Asn	Leu	Leu
10	Glu 865	Asn	Val	Ser	Asp	Glu 870	Leu	Tyr	Glu	Leu	Lys 875	Lys	Leu	Asn	Asn	Leu 880
15	Asp	Glu	Lys	Tyr	Leu 885	Ile	Ser	Phe	Glu	Asp 890	Ile	Ser	Lys	Asn	Asn 895	Ser
•	Thr	Tyr	Ser	Val 900	Arg	Phe	Ile	Asn	Lys 905	Ser	Asn	Gly	Glu	Ser 910	Val	Tyr
20	Val	Glu	Thr 915	Glu	Lys	Glu	Ile	Phe 920	Ser	Lys	Tyr	Ser	Glu 925	His	Ile	Thr
	Lys	Glu 930	Ile	Ser	Thr	Ile	Lys 935	Asn	Ser	Ile	Ile	Thr 940	Asp	Val	Asn	Gly
25	Asn 945	Leu	Leu	Asp	Asn	11e 950	Gln	Leu	Asp	His	Thr 955	Ser	Gln	Val	Asn	Thr 960
30	Leu	Asn	Ala	Ala	Phe 965	Phe	Ile	Gln	Ser	Leu 970	Ile	Asp	Tyr	Ser	Ser 975	Asn
	Lys	Asp	Val	Leu 980	Asn	Asp	Leu	Ser	Thr 985	Ser	Val	Lys	Val	Gln 990	Leu	Tyr
35	Ala	Gln	Leu 995	Phe	Ser	Thr	Gly	Leu 1000		Thr	Ile	Tyr	Asp 1009		Ile	Gln
	Leu	Val 1010	Asn)	Leu	lle	Ser	Asn 1015		Val	Asn	Λsp	Thr 1020		Asn	Val	Leu
10	Pro 1025	Thr	Ile	Thr	Glu	Gly 1030	Ile	Pro	Ile	Val	Ser 1035		Ile	Leu	Asp	Gly 1040
4 5	Ile	Asn	Leu	Gly	Ala 1049		Ile	Lys	Glu	Leu 1050		Asp	Glu	His	Asp 1055	
	Leu	Leu	Lys	Lys 1060	Glu)	Leu	Glu	Ala	Lys 1065		Gly	Val	Leu	Ala 1070		Asn
50	Met	Ser	Leu 1079		Ile	Ala	Ala	Thr 1080		Ala	Ser	Ile	Val 1085		Ile	Gly
	Ala	Glu 1090	Val	Thr	Ile	Phe	Leu 1095		Pro	Ile	Ala	Gly 1100		Ser	Ala	Gly
55	Ile 1105	Pro	Ser	Leu	Val	Asn 1110	Asn)	Glu	Leu	Ile	Leu 1115		Asp	Lys	Ala	Thr 1120
50	Ser	Val	Val	Asn	Tyr 1125	Phe	Asn	His	Leu	Ser 1130	Glu	Ser	Lys	Lys	Tyr 1135	
	Pro	Leu	Lys	Thr 1140	Glu	qaA	Asp	Lys	Ile 1145		Val	Pro	Ile	Asp 1150		Leu
55	Val	Ile	Ser 1155	Glu	Ile	Ąsp	Phe	Asn 1160		Asn	Ser	Ile	Lys 1165		Gly	Thr
	Cys	Asn 1170	Ile	Leu	Ala	Met	Glu 1175	Gly	Glγ	Ser	Gly	His 1180		Val	Thr	Gly

	As. 11	n I 85	le	Asp	Hi:	s Phe	e Phe 119	e Sei 90	Se	r Pro	Se:	r Ile 119	e Se:	r Se	r Hi	s Ile	Pro
, 5	Se	r I.	eu	Ser	116	€ Tyı 120	r Ser 05	Ala	Ile	≘ Gly	/ Ile	≘ Glu 10	ı Thi	r Gl	u Ası	1 Let	ı Asp
	Phe	e s	er	Lys	Lys 122	5 Ile 20	e Met	Met	Let	Pro 122	Asr	n Ala	Pro	Se:	r Arg 123	y Val	Phe
10	Tr	Т	rp	Glu 123	Thr 5	Gly	/ Ala	Val	Pro	Gly	' Lei	a Arg	Ser	Let 124	1 Glu	ı Asr	Asp
15	Gly	/ T	hr 250	Arg	Leu	l Leu	Asp	Ser 125	Ile 5	Arg	Asp	Leu	Tyr 126	P1=0	Gly	' Lys	Phe
	Ту1 126	т 55	rp	Arg	Phe	Tyr	Ala 127	Phe 0	Phe	Asp	Tyr	Ala 127	Ile 5	Thr	Thr	Leu	Lys 1280
20	Pro	V.	al	Туг	Glu	128	Thr 5	Asn	lle	Lys	Ile 129	Lys 0	Leu	Asp	Lys	Asp	Thr
	Arg	A:	sn	Phe	11e 130	Met O	Pro	Thr	Ile	Thr 130	Thr 5	Asn	Glu	Ile	Arg		Lys
25	Leu	S€	r	Tyr 131	Ser 5	Phe	Asp	Gly	Ala 132	Gly 0	Gly	Thr	Tyr	Ser 132	Leu 5	Leu	Leu
30								- 23.	,				134	o			Leu
							133(,				135	5				Asn 1360
35						130.					13/0	U				137	5
10					+50		Lys			1385	•				139)	
40							Asn		1400	,				140	5		
45							Ser	1415	,				1420)			
•							Leu 1430					1435)				1440
50						1443					1450)				1455	, ·
22						•	Tyr			1465					1470)	
55							Ser		1400	'				1485	•		
60			- •					1475					1500				
							Ala 1510					1515					1520
65						1323					1530					1535	
	Ser	Ile	≘ A	sp :	Phe 1540	Ser	Ile	Ser .	Leu	Val 1545	Ser	Lys	Asn	Gln	Val 1550		Val

	Asr	ı Gl	y Let 15	u Tyr 55	Leu	ı Asr	Glu	1 Set 156	val	Туг	Ser	Ser	Tyr 156		ı Asp	Phe
5	Va]	l Ly 15	s Ası 70	n Ser	Asp	Gly	/ His	His	as Asn	Thr	Ser	Asn 158	Phe	Met	Asn	Leu
	Phe 158	e Le 35	u Ası) Asn	Ile	Ser 159	Phe	Trp	Lys	Leu	Phe	Gly 5	Phe	'Glu	Asn	Ile 1600
10	Asn	Ph	e Val	l Ile	Asp 160	Lys 5	Tyr	Phe	Thr	Leu 161	Val	Gly	Lys	Thr	Asn 161	
15	Gly	ту:	r Va]	Glu 162	Phe 0	Ile	Cys	Asp	Asn 162	Asn 5	Lys	Asn	Ile	Asp 163		Tyr
	Phe	Gl	y Glu 163	Trp	Lys	Thr	Ser	Ser 164	Ser 0	Lys	Ser	Thr	Ile 164	Phe 5	Ser	Gly
20	Asn	Gly 16	y Arg 50	Asn	Val	Val	Val 165	Glu 5	Pro	Ile	Tyr	Asn 166	Pro 0	Asp	Thr	Gly
	Glu 166	Asp 5	o Ile	Ser	Thr	Ser 167	Leu 0	Asp	Phe	Ser	Tyr 167	Glu 5	Pro	Leu	Tyr	Gly 1680
25	Ile	Asp	Arg	Tyr	Ile 168	Asn 5	Lys	Val	Leu	Ile 169	Ala O	Pro	Asp	Leu	Tyr 169	
30	Ser	Leu	ılle	Asn 170	lle	Asn	Thr	Asn	Tyr 170	Туг 5	Ser	Asn	Glu	Tyr 1710	Tyr	Pro
	Glu	Ile	11e	Val 5	Leu	Asn	Pro	Asn 172	Thr 0	Phe	His	Lys	Lys 1725	Val	Asn	Ile
35		1/3		Ser			173	5				1740)			
	Asp 174	Phe 5	lle	Leu	Val	Arg 1750	Tyr	Leu	Glu	Glu	Ser 1755	Asn	Lys	Lys	Ile	Leu 1760
40				Arg	1/65	•				1770)				1779	5
45				Ile 1780	,				1/85	l				1790)	
t			1/9					1800)				1805			•
50		101	U	Gly			1815	•				1820				-
· •	102.	,		Lys		1830					1835					1840
55					1845					1850	t				1855	
50				Lys 1860					1865					1870		
			10/5					1880					1885			_
55		103	J	Gln			1895					1900				
	Phe 1905	Ala	Pro	Ala .	Asn '	Thr 1910	Gln	Asn	Asn .	Asn	Ile 1915	Glu (Gly	Gln .		Ile 1920

Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Asp Asn Asn Ser Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Glu 5 Lys Tyr Tyr Phe Asn Pro Asn Asn Ala Ile Ala Ala Val Gly Leu Gln 1960 10 Val Ile Asp Asn Asn Lys Tyr Tyr Phe Asn Pro Asp Thr Ala Ile Ile 1975 1980 Ser Lys Gly Trp Gln Thr Val Asn Gly Ser Arg Tyr Tyr Phe Asp Thr 1995 15 Asp Thr Ala Ile Ala Phe Asn Gly Tyr Lys Thr Ile Asp Gly Lys His Phe Tyr Phe Asp Ser Asp Cys Val Val Lys Ile Gly Val Phe Ser Thr 20 Ser Asn Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Tyr Asn Asn Asn 2040 25 lle Glu Gly Gln Ala Ile Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn 2055 Gly Lys Lys Tyr Tyr Phe Asp Asn Asn Ser Lys Ala Val Thr Gly Leu 30 Gln Thr Ile Asp Ser Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Glu 2090 Ala Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn 35 2100 Thr'Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys 2115 2120 40 Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Ile Ala Ser Thr Gly Tyr Thr 2135 Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Thr Asp Gly Ile Met Gln 2150 2155 45 lle Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala Pro Ala 2170 Asn Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile Leu Tyr Gln Asn 50 2185 Glu Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Gly Ser Asp Ser 55 Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Lys Lys Tyr Tyr Phe Asn Pro Asn Asn Ala Ile Ala Ala Ile His Leu Cys Thr Ile Asn Asn 2230 60 Asp Lys Tŷr Tyr Phe Ser Tyr Asp Gly Ile Leu Gln Asn Gly Tyr Ile Thr Ile Glu Arg Asn Asn Phe Tyr Phe Asp Ala Asn Asn Glu Ser Lys 65 Met Val Thr Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala 2280

	Pro	22	a Ası 90	n Thi	c His	s Asr	229	n Asr 95	ı Ile	e Gl	u Gly	/ Glr 230	n Ala	a Il	e Val	l Tyr
5	Glr 230	n A s:	n Ly	s Phe	? Lev	Thr 231	Let .0	ı Asr	ı Gly	y Lys	E Lys 231	. Туг .5	ту	r Ph	e Asp	Asn 2320
	Asp	Se:	r Lys	s Ala	Val 232	Thr	Gly	/ Trp	Glr	Thi 233	lle 30	Asp	Gly	/ Ly:	s Lys 233	Tyr
10	Туг	Phe	e Asr	1 Leu 234	Asn O	Thr	Ala	Glu	Ala 234	Ala 15	Thr	Gly	Trp	Gl: 23!	n Thr 50	Ile
15	Asp	Gly	235	Lys 55	Tyr	Tyr	Phe	236	Lei 0	ı Asn	Thr	Ala	Glu 236	Ala 5	a Ala	Thr
	Gly	237	o Gln 70	Thr	Ile	Asp	Gly 237	Lys 5	Lys	туг	Tyr	Phe 238	Asn 0	Thi	. Asn	Thr
20	Phe 238	11€ 5	Ala	Ser	Thr	Gly 239	Tyr 0	Thr	Ser	Ile	Asn 239	Gly 5	Lys	His	s Phe	Tyr 2400
.					440	5				241	0				Pro 241	5
25				242	U				242	5				243		
30	Gly	Gln	Ala 243	Ile 5	Leu	Tyr	Gln	Asn 244	Lys 0	Phe	Leu	Thr	Leu 244		Gly	Lys
		243	Ÿ				245	5				246	0		Arg	
35	240	_				24/(J				2475	5			Ala	2480
40					248	•				249	0				Thr 2495	5
40				2500	,				250	5				251		
45			201;	•				2520)				2525	5	Gly	
•		200	J				2535	>				2540)		Asn	
50	234.	,				2550	,				2555				His	2560
55					2565)				2570)				Trp 2575	ı
, ,				2580	1				2585	i				2590		
5()			2333	,				2600					2605		Arg	
		2010	,				2615					2620			Glu	
5	2023					2630					2635					2640
	arg	ryr	GIn	Asn .	Arg 2645	Phe :	Leu	His :	Leu	Leu 2650	Gly :	Lys	Ile	Tyr	Tyr 2655	Phe

Gly Asn Asn Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asn Gly Lys 2665 Val Tyr Tyr Phe Met Pro Asp Thr Ala Met Ala Ala Ala Gly Gly Leu . 5 Phe Glu Ile Asp Gly Val Ile Tyr Phe Phe Gly Val Asp Gly Val Lys 10 Ala Pro Gly Ile Tyr Gly 2705 (2) INFORMATION FOR SEQ ID NO:7: 15 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 811 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: unknown (D) TOPOLOGY: unknown 20 (ii) MOLECULE TYPE: protein (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7: 25 Ser Tyr Lys Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Asn Asp Gly Val Met Gln Leu Gly Val Phe Lys Gly Pro Asp Gly Phe Glu Tyr Phe 30 Ala Pro Ala Asn Thr Gln Asn Asn Ile Glu Gly Gln Ala Ile Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Asp 35 Asn Asn Ser Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Glu Lys 40 Tyr Tyr Phe Asn Pro Asn Asn Ala Ile Ala Ala Val Gly Leu Gln Val Ile Asp Asn Asn Lys Tyr Tyr Phe Asn Pro Asp Thr Ala Ile Ile Ser 105 45 Lys Gly Trp Gln Thr Val Asn Gly Ser Arg Tyr Tyr Phe Asp Thr Asp Thr Ala Ile Ala Phe Asn Gly Tyr Lys Thr Ile Asp Gly Lys His Phe 50 Tyr Phe Asp Ser Asp Cys Val Val Lys Ile Gly Val Phe Ser Thr Ser 55 Asn Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Tyr Asn Asn Asn Ile Glu Gly Gln Ala Ile Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly 60 Lys Lys Tyr Tyr Phe Asp Asn Asn Ser Lys Ala Val Thr Gly Leu Gln Thr Ile Asp Ser Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Glu Ala 65 Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr 230 235

•	Asn	Thr	Ala	Glu	Ala 245	Ala	Thr	Gly	Trp	Gln 250	Thr	Ile	Asp	Gly	Lys 255	-
5	Tyr	Туr	Phe	Asn 260	Thr	Asn	Thr	Ala	Ile 265	Ala	Ser	Thr	Gly	Tyr 270		Ile
	Ile	Asn	Gly 275	Lys	His	Phe	Tyr	Phe 280	Asn	Thr	Asp	Gly	Ile 285	Met	Gln	Ile
10	Gly	Val 290	Phe	Lys	Gly	Pro	Asn 295	Gly	Phe	Glu	Tyr	Phe 300	Ala	Pro	Ala	Asn
15	Thr 305	Asp	Ala	Asn	Asn	Ile 310	Glu	Gly	Gln	Ala	Ile 315	Leu	Tyr	Gln	Asn	Glu 320
	Phe	Leu	Thr	Leu	Asn 325	Gly	Lys	Lys	Tyr	Tyr 330	Phe	Gly	Ser	Asp	Ser 335	Lys
20	Ala	Val	Thr	Gly 340	Trp	Arg	Ile	Ile	Asn 345	Asn	Lys	Lys	Tyr	Tyr 350	Phe	Asn
	Pro	Asn	Asn 355	Ala	Ile	Ala	Ala	Ile 360	His	Leu	Cys	Thr	Ile 365	Asn	Asn	Asp
25	Lys	Tyr 370	Tyr	Phe	Ser	Tyr	Asp 375	Gly	Ile	Leu	Gln	Asn 380	Gly	туг	Ile	Thr
30	Ile 385	Glu	Arg	Asn	Asn	Phe 390	Tyr	Phe	Asp	Ala	Asn 395	Asn	Glu	Ser	Lys	Met 400
	Val	Thr	Gly	Val	Phe 405	Lys	Gly	Pro	Asn	Gly 410	Phe	Glu	Tyr	Phe	Ala 415	Pro
35	Ala	Asn	Thr	His 420	Asn	Asn	Asn	Ile	Glu 425	Gly	Gln	Ala	Ile	Val 430	Tyr	Gln
	Asn	Lys	Phe 435	Leu	Thr	Leu	Asn	Gly 440	Lys	Lys	Tyr	Tyr	Phe 445	Asp	Asn	Asp
40	Ser	Lys 450	Ala	Val	Thr	Gly	Trp 455	Gln	Thr	Ile	Asp	Gly 460	Lys	Lys	Tyr	Tyr
45	Phe 465	Asn	Leu	Asn	Thr	Ala 470	Glu	Ala	Ala	Thr	Gly 475	Trp	Gln	Thr	Ile	Asp 480
	Gly	Lys	Lys	туr	Tyr 485	Phe	Asn	Leu	Asn	Thr 490	Ala	Glu	Ala	Ala	Thr 495	Gly
50	Trp	Gln	Thr	Ile 500	Asp	Gly	Lys	Lys	Tyr 505	Tyr	Phe	Asn	Thr	Asn 510	Thr	Phe
	Ile	Ala	Ser 515	Thr	Gly	Tyr	Thr	Ser 520	Ile	Asn	Gly	Lys	His 525	Phe	Tyr	Phe
55	Asn	Thr 530	Asp	Gly	Ile	Met	Gln 535	Ile	Gly	Val	Phe	Lys 540	Gly	Pro	Asn	Gly
60	Phe 545	Glu	Tyr	Phe	Ala	Pro 550	Ala	Asn	Thr	Asp	Ala 555	Asn	Asn	Ile	Glu	Gly 560
	Gln	Ala	Ile	Leu	Tyr 565	Gln	Asn	Lys	Phe	Leu 570	Thr	Leu	Asn	Gly	Lys 575	Lys
65	Tyr	Tyr	Phe	Gly 580	Ser	Asp	Ser	Lys	Ala 585	Val	Thr	Glγ	Leu	Arg 590	Thr	Ile
	Asp	Gly	Lys 595	Lys	Tyr	Tyr	Phe	Asn 600	Thr	Asn	Thr	Ala	Val 605	Ala	Val	Thr

,		Gly	7 Trp	Gln	Thr	Ile	Asn	Gly 615	Lys	Lys	туг	Tyr	Phe 620	Asn	Thr	Asn	Thr
5		Ser 625	Ile	Ala	Ser	Thr	Gly 630	Tyr	Thr	Ile	Ile	Ser 635	Gly	Lys	His	Phe	Tyr 640
		Phe	Asn	Thr	Asp	Gly 645	Ile	Met	Gln	Ile	Gly 650	Val	Phe	Lys	Gly	Pro 655	
10		Gly	Phe	Glu	Tyr 660	Phe	Ala	Pro	Ala	Asn 665	Thr	Asp	Ala	Asn	Asn 670	Ile	Glu
15		Gly	Gln	Ala 675	Ile	Arg	Tyr	Gln	Asn 680	Arg	Phe	Leu	Tyr	Leu 685	His	Asp	Asn
		Ile	Tyr 690	туг	Phe	Gly	Asn	Asn 695	Ser	Lys	Ala	Ala	Thr 700	Gly	Trp	Val	Thr
20		Ile 705	Asp	Gly	Asn	Arg	Tyr 710	Tyr	Phe	Glu	Pro	Asn 715	Thr	Ala	Met	Gly	Ala 720
		Asn	Gly	Tyr	Lys	Thr 725	Ile	Asp	Asn	Lys	Asn 730	Phe	Tyr	Phe	Arg	Asn 735	Gly
25		Leu	Pro	Gļn	Ile 740	Gly	Val	Phe	Lys	Gly 745	Ser	Asn	Gly	Phe	Glu 750	Tyr	Phe
30		Ala	Pro	Ala 755	Asn	Thr	Asp	Ala	Asn 760	Asn	Ile	Glu	Gly	Gln 765	Ala	Ile	Arg
		Tyr	Gln 770	Asn	Arg	Phe	Leu	His 775	Leu	Leu	Gly	Lys	Ile 780	Tyr	Tyr	Phe	Gly
35		Asn 785	Asn	Ser	Lys	Ala	Val 790	Thr	Gly	Trp	Gln	Thr 795	Ile	Asn	Gly	Lys	Val 800
		Tyr	Tyr	Phe	Met	Pro 805	Asp	Thr	Ala	Met	Ala 810	Λla					
40	(2)	INFO	RMATI	ON F	OR S	EQ I	D NO	8:									
45		(i)	(B)	LEN TYF STR	CHA IGTH: E: a IANDE POLOG	91 mino DNES	amin aci S: u	o ac d nkno	ids								
		(ii)	MOLE	CULE	TYP	E: p	rote	in									
50		(xi)	SEQU	ENCE	DES	CRIP	TION	: SE	Q ID	NO:	8 :						
		Ser 1	Tyr	Lys	lle	Ile 5	Asn	Gly	Lys	His	Phe 10	туг	Phe	Asn	Asn	Asp 15	Gly
55		Val	Met	Gln	Leu 20	Gly	Val	Phe	Lys	Gly 25	Pro	Asp	Gly	Phe	Glu 30	Tyr	Phe
60		Ala	Pro	Ala 35	Asn	Thr	Gln	Asn	Asn 40	Asn	Ile	Glu		Gln 45	Ala	Ile	Val
		Tyr	Gln 50	Ser	Lys	Phe	Leu	Thr 55	Leu	Asn	Gly	Lys	Lys 60	Tyr	Tyr	Phe	Asp
65		Asn 65	Asn	Ser	Lys	Ala	Val 70	Thr	Gly	Trp	Arg	Ile 75	Ile .	Asn	Asn	Glu	Lys 80
		Tyr	Tyr	Phe		Pro 85	Asn	Asn .	Ala		Ala 90	Ala					

(2) INFORMATION FOR SEQ ID NO:9: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 7101 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) 10 (ix) FEATURE: (A) NAME/KEY: CDS (B) LOCATION: 1..7098 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9: ATG AGT TTA GTT AAT AGA AAA CAG TTA GAA AAA ATG GCA AAT GTA AGA 48 Met Ser Leu Val Asn Arg Lys Gln Leu Glu Lys Met Ala Asn Val Arg 20 TTT CGT ACT CAA GAA GAT GAA TAT GTT GCA ATA TTG GAT GCT TTA GAA Phe Arg Thr Gln Glu Asp Glu Tyr Val Ala Ile Leu Asp Ala Leu Glu 96 25 GAA TAT CAT AAT ATG TCA GAG AAT ACT GTA GTC GAA AAA TAT TTA AAA 144 Glu Tyr His Asn Met Ser Glu Asn Thr Val Val Glu Lys Tyr Leu Lys TTA AAA GAT ATA AAT AGT TTA ACA GAT ATT TAT ATA GAT ACA TAT AAA Leu Lys Asp Ile Asn Ser Leu Thr Asp Ile Tyr Ile Asp Thr Tyr Lys 192 30 AAA TCT GGT AGA AAT AAA GCC TTA AAA AAA TTT AAG GAA TAT CTA GTT 240 Lys Ser Gly Arg Asn Lys Ala Leu Lys Lys Phe Lys Glu Tyr Leu Val 35 ACA GAA GTA TTA GAG CTA AAG AAT AAT AAT TTA ACT CCA GTT GAG AAA 28R Thr Glu Val Leu Giu Leu Lys Asn Asn Leu Thr Pro Val Glu Lys 40 AAT TTA CAT TTT GTT TGG ATT GGA GGT CAA ATA AAT GAC ACT GCT ATT 336 Asn Leu His Phe Val Trp Ile Gly Gly Gln Ile Asn Asp Thr Ala Ile 100 105 45 AAT TAT ATA AAT CAA TGG AAA GAT GTA AAT AGT GAT TAT AAT GTT AAT 384 Asn Tyr Ile Asn Gln Trp Lys Asp Val Asn Ser Asp Tyr Asn Val Asn GTT TTT TAT GAT AGT AAT GCA TTT TTG ATA AAC ACA TTG AAA AAA ACT 432 50 Val Phe Tyr Asp Ser Asn Ala Phe Leu Ile Asn Thr Leu Lys Lys Thr GTA GTA GAA TCA GCA ATA AAT GAT ACA CTT GAA TCA TTT AGA GAA AAC 480 Val Val Glu Ser Ala Ile Asn Asp Thr Leu Glu Ser Phe Arg Glu Asn 55 145 155 TTA AAT GAC CCT AGA TTT GAC TAT AAT AAA TTC TTC AGA AAA CGT ATG Leu Asn Asp Pro Arg Phe Asp Tyr Asn Lys Phe Phe Arg Lys Arg Met 528 60 GAA ATA ATT TAT GAT AAA CAG AAA AAT TTC ATA AAC TAC TAT AAA GCT 576 Glu Ile Ile Tyr Asp Lys Gln Lys Asn Phe Ile Asn Tyr Tyr Lys Ala 185 CAA AGA GAA GAA AAT CCT GAA CTT ATA ATT GAT GAT ATT GTA AAG ACA 65 624 Gln Arg Glu Glu Asn Pro Glu Leu Ile Ile Asp Asp Ile Val Lys Thr

205

200

195

	TAT	CTT Leu 210	ser	AAT Asn	GAG Glu	TAT Tyr	TCA Ser 215	Lys	GAG Glu	ATA Ile	GAT Asp	GAA Glu 220	CTT Leu	AAT Asn	ACC Thr	TAT Tyr	672
5	ATT Ile 225	GAA Glu	GAA Glu	TCC Ser	TTA Leu	AAT Asn 230	AAA Lys	ATT Ile	ACA Thr	CAG Gln	AAT Asn 235	AGT Ser	GGA Gly	AAT Asn	GAT Asp	GTT Val 240	720
10	nr 9	ASII	Pile	Giu	245	TTT Phe	Lys	Asn	Gly	Glu 250	Ser	Phe	Asn	Leu	Tyr 255	Glu	768
15	GIII	Gru	Leu	260	GIU	AGG Arg	Trp	Asn	265	Ala	Ala	Ala	Ser	Asp 270	Ile	Leu	816
20	AGA Arg	ATA lle	TCT Ser 275	GCA Ala	TTA Leu	Lys	GAA Glu	ATT Ile 280	GGT Gly	GGT Gly	ATG Met	TAT Tyr	TTA Leu 285	GAT Asp	GTT Val	GAT Asp	864
	ATG Met	TTA Leu 290	CCA Pro	GGA Gly	ATA Ile	CAA Gln	CCA Pro 295	GAC Asp	TTA Leu	TTT Phe	GAG Glu	TCT Ser 300	ATA Ile	GAG Glu	AAA Lys	CCT Pro	912
25	AGT Ser 305	TCA Ser	GTA Val	ACA Thr	GTG Val	GAT Asp 310	TTT Phe	TGG Trp	GAA Glu	ATG Met	ACA Thr 315	AAG Lys	TTA Leu	GAA Glu	GCT Ala	ATA Ile 320	960
30	ATG Met	AAA Lys	TAC Tyr	AAA Lys	GAA Glu 325	TAT Tyr	ATA Ile	CCA Pro	GAA Glu	TAT Tyr 330	ACC Thr	TCA Ser	GAA Glu	CAT His	TTT Phe 335	GAC Asp	1008
35	Mec	Leu	Asp	340	GLu	GTT Val	Gln	Ser	Ser 345	Phe	Glu	Ser	Val	Leu 350	Ala	Ser	1056
40	AAG' Lys	TCA Ser	GAT Asp 355	AAA Lys	TCA Ser	GAA Glu	ATA Ile	TTC Phe 360	TCA Ser	TCA Ser	CTT Leu	GGT Gly	GAT Asp 365	ATG Met	GAG Glu	GCA Ala	1104
	TCA Ser	CCA Pro 370	CTA Leu	GAA Glu	GTT Val	AAA Lys	ATT Ile 375	GCA Ala	TTT Phe	AAT Asn	AGT Ser	AAG Lys 380	GGT Gly	ATT Ile	ATA Ile	AAT Asn	1152
45	CAA Gln 385	GGG Gly	CTA Leu	ATT Ile	TCT Ser	GTG Val 390	AAA Lys	GAC Asp	TCA Ser	TAT Tyr	TGT Cys 395	AGC Ser	AAT Asn	TTA Leu	ATA Ile	GTA Val 400	1200
50	AAA Lys	CAA Gln	ATC Ile	GAG Glu	AAT Asn 405	AGA Arg	Tyr	Lys	ATA Ile	Leu	Asn	AAT Asn	AGT Ser	TTA Leu	AAT Asn 415	CCA Pro	1248
55	GCT Ala	ATT Ile	AGC Ser	GAG Glu 420	GAT Asp	AAT Asn	GAT Asp	TTT Phe	AAT Asn 425	ACT Thr	ACA Thr	ACG Thr	AAT Asn	ACC Thr 430	TTT Phe	ATT Ile	1296
60	GAT Asp	AGT Ser	ATA Ile 435	ATG Met	GCT Ala	GAA Glu	GCT Ala	AAT Asn 440	GCA Ala	GAT Asp	AAT Asn	GGT Gly	AGA Arg 445	TTT Phe	ATG Met	ATG Met	1344
	GAA Glu	CTA Leu 450	GGA Gly	AAG Lys	TAT Tyr	TTA Leu	AGA Arg 455	GTT Val	GGT Gly	TTC Phe	TTC Phe	CCA Pro 460	GAT Asp	GTT Val	AAA Lys	ACT Thr	1392
65	ACT Thr 465	ATT Ile	AAC Asn	TTA Leu	AGT Ser	GGC Gly 470	CCT Pro	GAA Glu	GCA Ala	TAT Tyr	GCG Ala 475	GCA Ala	GCT Ala	TAT Tyr	CAA Gln	GAT Asp 480	1440

	TTA Leu	TTA Leu	ATG Met	TTT Phe	AAA Lys 485	GAA Glu	GGC Gly	AGT Ser	ATG Met	AAT Asn 490	ATC Ile	CAT His	TTG Leu	ATA Ile	GAA Glu 495	GCT Ala	1488
5	gat Asp	TTA Leu	AGA Arg	AAC Asn 500	TTT Phe	GAA Glu	ATC Ile	TCT Ser	AAA Lys 505	ACT Thr	AAT Asn	ATT	TCT Ser	CAA Gln 510	TCA Ser	ACT Thr	1536
10	GAA Glu	CAA Gln	GAA Glu 515	ATG Met	GCT Ala	AGC Ser	TTA Leu	TGG Trp 520	TCA Ser	TTT Phe	GAC Asp	GAT Asp	GCA Ala 525	AGA Arg	GCT Ala	AAA Lys	1584
15	GCT Ala	CAA Gln 530	TTT Phe	GAA Glu	GAA Glu	TAT Tyr	AAA Lys 535	AGG Arg	AAT Asn	TAT Tyr	TTT Phe	GAA Glu 540	GGT Gly	TCT Ser	CTT Leu	GGT Gly	1632
20	GAA Glu 545	GAT Asp	GAT Asp	AAT Asn	CTT Leu	GAT Asp 550	TTT Phe	TCT Ser	CAA Gln	AAT Asn	ATA Ile 555	GTA Val	GTT Val	GAC Asp	AAG Lys	GAG Glu 560	1680
	TAT Tyr	CTT Leu	TTA Leu	GAA Glu	AAA Lys 565	ATA Ile	TCT Ser	TCA Ser	TTA Leu	GCA Ala 570	AGA Arg	AGT Ser	TCA Ser	GAG Glu	AGA Arg 575	GGA Gly	1728
25	TAT Tyr	ATA Ile	CAC His	TAT Tyr 580	ATT Ile	GTT Val	CAG Gln	TTA Leu	CAA Gln 585	GGA Gly	GAT Asp	AAA Lys	ATT Ile	AGT Ser 590	TAT Tyr	GAA Glu	1776
30	GCA Ala	GCA Ala	TGT Cys 595	AAC Asn	TTA Leu	TTT Phe	GCA Ala	AAG Lys 600	ACT Thr	CCT Pro	TAT Tyr	GAT Asp	AGT Ser 605	GTA Val	CTG Leu	TTT Phe	1824
35	CAG Gln	AAA Lys 610	AAT Asn	ATA Ile	GAA Glu	GAT Asp	TCA Ser 615	GAA Glu	ATT Ile	GCA Ala	TAT Tyr	TAT Tyr 620	TAT Tyr	AAT Asn	CCT Pro	GGA Gly	1872
40	GAT Asp 625	GGT Gly	GAA Glu	ATA Ile	CAA Gln	GAA Glu 630	ATA Ile	GAC Asp	AAG Lys	TAT Tyr	AAA Lys 635	ATT Ile	CCA Pro	AGT Ser	ATA Ile	ATT Ile 640	1920
	TCT Ser	GAT Asp	AGA Arg	CCT Pro	AAG Lys 645	ATT Ile	AAA Lys	TTA Leu	ACA Thr	TTT Phe 650	ATT Ile	GGT Gly	CAT His	GG T Gly	AAA Lys 655	GAT Asp	1968
45	GAA Glu	TTT Phe	AAT Asn	ACT Thr 660	GAT Asp	ATA Ile	TTT Phe	GCA Ala	GGT Gly 665	TTT Phe	GAT Asp	GTA Val	GAT Asp	TCA Ser 670	TTA Leu	TCC Ser	2016
50	ACA Thr	GAA Glu	ATA Ile 675	GAA Glu	GCA Ala	GCA Ala	ATA Ile	GAT Asp 680	TTA Leu	GCT Ala	aaa Lys	GAG Glu	GAT Asp 685	ATT Ile	TCT Ser	CCT Pro	2064
55	AAG Lys	TCA Ser 690	ATA Ile	GAA Glu	ATA Ile	AAT Asn	TTA Leu 695	TTA Leu	GGA Gly	TGT Cys	AAT Asn	ATG Met 700	TTT Phe	AGC Ser	TAC Tyr	TCT Ser	2112
60	ATC Ile 705	AAC Asn	GTA Val	GAG Glu	GAG Glu	ACT Thr 710	TAT Tyr	CCT Pro	GGA Gly	AAA Lys	TTA Leu 715	TTA Leu	CTT Leu	AAA Lys	GTT Val	AAA Lys 720	2160
	GAT Asp	AAA Lys	ATA Ile	TCA Ser	GAA Glu 725	TTA Leu	ATG Met	CCA Pro	TCT Ser	ATA Ile 730	AGT Ser	CAA Gln	GAC Asp	TCT Ser	ATT Ile 735	ATA Ile	2208
65	GTA Val	AGT Ser	GCA Ala	AAT Asn 740	CAA Gln	TAT Tyr	GAA Glu	GTT Val	AGA Arg 745	ATA Ile	AAT Asn	AGT Ser	GAA Glu	GGA Gly 750	AGA Arg	AGA Arg	2256

	GAA Glu	TTA Leu	TTG Leu 755	Asp	CAT His	TCT Ser	GG T Gly	GAA Glu 760	Trp	ATA Ile	AAT Asn	AAA Lys	GAA Glu 765	GAA Glu	AGT Ser	ATT		2304
5	ATA Ile	AAG Lys 770	725	ATT Ile	TCA Ser	TCA Ser	AAA Lys 775	GAA Glu	TAT Tyr	ATA Ile	TCA Ser	TTT Phe 780	Asn	CCT	AAA Lys	GAA Glu		2352
10	785	2,3	116	1111	val	790	ser	гÀг	Asn	Leu	Pro 795	Glu	Leu	Ser	Thr	TTA Leu 800		2400
15	200	J	GIU	116	805	ASI	ASN	ser	Asn	Ser 810	Ser	Asp	Ile	Glu	Leu 815			2448
20	J. U	Dy3	vai	820	Leu	inr	GIU	Cys	825	Ile	Asn	Val	Ile	Ser 830	Asn			2496
	GAT Asp	ACG Thr	CAA Gln 835	ATT Ile	GTT Val	GAG Glu	GAA Glu	AGG Arg 840	ATT Ile	GAA Glu	GAA Glu	GCT Ala	AAG Lys 845	AAT Asn	TTA Leu	ACT Thr		2544
25	TCT Ser	GAC Asp 850	TCT Ser	ATT Ile	AAT Asn	TAT Tyr	ATA Ile 855	AAA Lys	GAT Asp	GAA Glu	TTT Phe	AAA Lys 860	CTA Leu	ATA Ile	GAA Glu	TCT Ser		2592
30	865	361	Asp	Ala	neu	870	Asp	Leu	Lys	Gln	Gln 875	Asn	GAA Glu	Leu	Glu	Asp 880		2640
35	501		FILE	116	885	Pne	Glu	Asp	Ile	Ser 890	Glu	Thr	GAT Asp	Glu	Gly 895	Phe		2688
40	DCI	116	Arg	900	116	ASN	rys	Glu	905	Gly	Glu	Ser	ATA Ile	Phe 910	Val	Glu		2736
	1111	Gru	915	1111	116	Pne	ser	920	Tyr	Ala	Asn	His	ATA Ile 925	Thr	Glu	Glu		2784
45	ATT Ile	TCT Ser 930	AAG Lys	ATA Ile	AAA Lys	GGT Gly	ACT Thr 935	ATA Ile	TTT Phe	GAT Asp	ACT Thr	GTA Val 940	AAT Asn	GGT Gly	AAG Lys	TTA Leu	;	2832
50	GTA Val 945	AAA Lys	AAA Lys	GTA Val	AAT Asn	TTA Leu 950	GAT Asp	ACT Thr	ACA Thr	CAC His	GAA Glu 955	GTA Val	AAT Asn	ACT Thr	TTA Leu	AAT Asn 960	:	2880
55	GCT Ala	GCA Ala	TTT Phe	TTT Phe	ATA Ile 965	CAA Gln	TCA Ser	TTA Leu	ATA Ile	GAA Glu 970	TAT Tyr	AAT Asn	AGT Ser	TCT Ser	AAA Lys 975	GAA Glu	:	2928
60	TCT Ser	CTT Leu	AGT Ser	AAT Asn 980	TTA Leu	AGT Ser	GTA Val	GCA Ala	ATG Met 985	AAA Lys	GTC Val	CAA Gln	GTT Val	TAC Tyr 990	GCT Ala	CAA Gln	:	2976
	TTA Leu	FILE	AGT Ser 995	ACT Thr	GGT Gly	TTA Leu	AAT Asn	ACT Thr 1000	He	ACA Thr	GAT Asp	GCA Ala	GCC Ala 1005	Lys	GTT Val	GTT Val	;	3024

	GAA Glu	TTA Leu 101	vaı	TCA Ser	ACT Thr	GCA Ala	TTA Leu 101	Asp	GAA Glu	ACT The	Γ ATA	GAC Asp	Leu	CTI Leu	CCT Pro	C ACA	3072
5	TTA Leu 1025	Set	GAA Glu	GGA Gly	TTA Leu	CCT Pro 103	He	ATT	GCA Ala	ACT Thr	T ATT	Ile	GAT Asp	GGT Gly	GTA Val	AGT Ser 1040	3120
10	TTA Leu	GGT Gly	GCA Ala	GCA Ala	ATC Ile 104	Lys	GAG Glu	CTA Leu	AGT Ser	GAA Glu 105	Thr	AGT Ser	GAC Asp	CCA Pro	TTA Leu 105	TTA Leu 5	3168
15	AGA Arg	CAA Gln	GAA Glu	ATA Ile 106	Glu	GCT Ala	AAG Lys	ATA Ile	GGT Gly 106	Ile	ATG Met	GCA Ala	GTA Val	AAT Asn 107	Leu	ACA Thr	3216
20	ACA Thr	GCT Ala	ACA Thr 107	ing	GCA Ala	ATC Ile	ATT Ile	ACT Thr 108	Ser	TCT Ser	TTG Leu	GGG Gly	ATA Ile 108	Ala	AGT Ser	GGA Gly	3264
	rne	1090	1.16	Leu	Leu	vai	1099	Leu	Ala	Gly	ATT Ile	Ser	Ala O	Gly	Ile	Pro	3312
25	1103	neu.	vai	ASII	Asn	1110	Leu)	Val	Leu	Arg	Asp 1119	Lys 5	Ala	Thr	Lys	Val 1120	3360
30	GTA (GAT Asp	TAT Tyr	TTT Phe	AAA Lys 112	His	GTT Val	TCA Ser	TTA Leu	GTT Val 113	Glu	ACT Thr	GAA Glu	GGA Gly	GTA Val 113	Phe	3408
35	ACT Thr	TTA Leu	TTA Leu	GAT Asp 1140	Asp	AAA Lys	ATA Ile	ATG Met	ATG Met 1149	Pro	CAA Gln	GAT Asp	GAT Asp	TTA Leu 1150	Val	ATA Ile	3456
40	TCA (31 U	11e 1155	Asp	Phe	Asn	Asn	Asn 1160	Ser	Ile	Val	Leu	Gly 1165	Lys	Cys	Glu	3504
	ATC 1	rgg Frp 1170	Arg	ATG Met	GAA Glu	GGT Gly	GGT Gly 1175	Ser	GGT Gly	CAT His	ACT Thr	GTA Val 1180	Thr	GAT Asp	GAT Asp	ATA Ile	3552
45	GAT (Asp F 1185	CAC His	TTC Phe	TTT Phe	TCA Ser	GCA Ala 1190	Pro	TCA Ser	ATA Ile	ACA Thr	TAT Tyr 1195	Arg	GAG Glu	CCA Pro	CAC H1s	TTA Leu 1200	3600
50	TCT A	ııe	ıyr	Asp	Va1 1205	Leu	Glu	Val	Gln	Lys 1210	Glu)	Glu	Leu	Asp	Leu 1215	Ser	3648
55	AAA (SAT S	Leu	ATG Met 1220	Val	TTA Leu	CCT . Pro .	Asn	GCT Ala 1225	Pro	AAT Asn	AGA Arg	Val	TTT Phe 1230	Ala	TGG Trp	3696
60	GAA A Glu T	. 111 (GGA Gly 1235	TGG . Trp	ACA Thr	CCA Pro	GIY :	TTA Leu 1240	Arg	AGC Ser	TTA Leu	Glu	AAT Asn 1245	GAT Asp	GGC Gly	ACA Thr	3744
	AAA C Lys L 1	TG ' eu i 250	TTA . Leu .	GAC (Asp	CGT Arg	TIE.	AGA (Arg) 1255	GAT . Asp .	AAC Asn	TAT Tyr	Glu	GGT Gly 1260	GAG Glu	TTT Phe	ТАТ Туг	TGG Trp	3792
65	AGA T Arg T 1265	AT : 'yr 1	Phe	GCT 1 Ala 1	Pne	ATA (Ile / 1270	GCT (Ala /	GAT (GCT Ala	Leu	ATA . Ile ' 1275	ACA Thr	ACA :	TTA /	Lys	CCA Pro 1280	3840

	AGA TAT GAA Arg Tyr Glu	GAT ACT AAT ATA Asp Thr Asn Ile 1285	A AGA ATA AAT 1 Arg Ile Asn I 1290	TTA GAT AGT AAT Leu Asp Ser Asn	ACT AGA 3888 Thr Arg 1295
.5	AGT TTT ATA Ser Phe Ile	GTT CCA ATA ATA Val Pro Ile Ile 1300	A ACT ACA GAA 1 Thr Thr Glu 1 1305	FAT ATA AGA GAA Fyr Ile Arg Glu 1310	Lys Leu
10	TCA TAT TCT Ser Tyr Ser 1315	TTC TAT GGT TCA Phe Tyr Gly Ser 5	A GGA GGA ACT T Gly Gly Thr T 1320	TAT GCA TTG TCT Tyr Ala Leu Ser 1325	CTT TCT 3984 Leu Ser
15	CAA TAT AAT Gln Tyr Asn 1330	ATG GGT ATA AAT Met Gly Ile Asn 133	ı Ile Glu Leu S	AGT GAA AGT GAT Ser Glu Ser Asp 1340	GTT TGG 4032 Val Trp
20	ATT ATA GAT Ile Ile Asp 1345	GTT GAT AAT GTT Val Asp Asn Val 1350	. Val Arg Asp V	GTA ACT ATA GAA Val Thr Ile Glu .355	TCT GAT 4080 Ser Asp 1360
	AAA ATT AAA Lys Ile Lys	AAA GCT GAT TTA Lys Gly Asp Leu 1365	ATA GAA GGT A I Ile Glu Gly I 1370	ATT TTA TCT ACA le Leu Ser Thr	CTA AGT 4128 Leu Ser 1375
25	ATT GAA GAG ile Glu Glu	AAT AAA ATT ATC Asn Lys Ile Ile 1380	TTA AAT AGC C Leu Asn Ser H 1385	CAT GAG ATT AAT His Glu Ile Asn 1390	Phe Ser
30	GGT GAG GTA Gly Glu Val 1395	AAT GGA AGT AAT Asn Gly Ser Asn 5	GGA TTT GTT T Gly Phe Val S 1400	CT TTA ACA TTT Ser Leu Thr Phe 1405	TCA ATT 4224 Ser Ile
35	TTA GAA GGA Leu Glu Gly 1410	ATA AAT GCA ATT Ile Asn Ala Ile 141	: Ile Glu Val A	SAT TTA TTA TCT SSP Leu Leu Ser 1420	AAA TCA 4272 Lys Ser
40	TAT AAA TTA Tyr Lys Leu 1425	CTT ATT TCT GGC Leu Ile Ser Gly 1430	Glu Leu Lys I	TA TTG ATG TTA le Leu Met Leu .435	AAT TCA 4320 Asn Ser 1440
	AAT CAT ATT Asn His Ile	CAA CAG AAA ATA Gln Gln Lys Ile 1445	GAT TAT ATA G Asp Tyr Ile G 1450	GA TTC AAT AGC ly Phe Asn Ser	GAA TTA 4368 Glu Leu 1455
45	CAG AAA AAT Gln Lys Asn	ATA CCA TAT AGC Ile Pro Tyr Ser 1460	TTT GTA GAT A Phe Val Asp S 1465	GT GAA GGA AAA er Glu Gly Lys 1470	Glu Asn
50	GGT TTT ATT Gly Phe Ile 1475	AAT GGT TCA ACA Asn Gly Ser Thr	AAA GAA GGT T Lys Glu Gly L 1480	TA TTT GTA TCT eu Phe Val Ser 1485	GAA TTA 4464 Glu Leu
55	CCT GAT GTA Pro Asp Val 1490	GTT CTT ATA AGT Val Leu Ile Ser 149	Lys Val Tyr M	ATG GAT GAT AGT let Asp Asp Ser 1500	AAG CCT 4512 Lys Pro
60	TCA TTT GGA Ser Phe Gly 1505	TAT TAT AGT AAT Tyr Tyr Ser Asn 1510	Asn Leu Lys A	AT GTC AAA GTT asp Val Lys Val 515	ATA ACT 4560 Ile Thr 1520
****	AAA GAT AAT Lys Asp Asn	GTT AAT ATA TTA Val Asn Ile Leu 1525	ACA GGT TAT T Thr Gly Tyr T 1530	TAT CTT AAG GAT Tyr Leu Lys Asp	GAT ATA 4608 Asp lle 1535
65	AAA ATC TCT Lys Ile Ser	CTT TCT TTG ACT Leu Ser Leu Thr 1540	CTA CAA GAT G Leu Gln Asp G 1545	GAA AAA ACT ATA Glu Lys Thr Ile 1550	Lys Leu

r	AA1 Asr	AG1 Sei	GT(Val 155	His	TTA Leu	GAT Asp	GAA Glu	AGT Ser 156	: Gly	A GTA Val	GCT Ala	r GAG a Glu	ATT	: Leu	AAC Lys	TTC Phe	4704
5	Med	157	0	Lys	: GI	/ Asn	157	Asn 5	Thr	Ser	Asp	Ser 158	Leu 0	Met	Ser	TTT Phe	4752
10	158	5	Ser	Met	Asn	11e 159	Lys 0	Ser	lle	Phe	Val 159	Asn 5	Phe	Leu	Gln	TCT Ser 1600	4800
15	· 1011	116	груѕ	Pne	11e 160	Leu 5	Asp	Ala	Asn	Phe 161	lle O	ATA Ile	Ser	Gly	Thr 161	Thr 5	4848
20	261	116	GIY	162	o Pne	GIU	Pne	ile	Cys 162	Asp 5	Glu	AAT Asn	Asp	Asn 163	Ile O	Gln	4896
'a-	FIO	lyt	163	5	Lys	РЛЕ	Asn	164	Leu 0	Glu	Thr	AAT Asn	Tyr 164	Thr 5	Leu	Tyr	4944
25	Veil	165	O ASII	arg	Gin	Asn	Met 165!	lle 5	Val	Glu	Pro	AAT Asn 1660	Tyr	Asp	Leu	Asp	4992
30	GAT Asp 166	Ser	GGA Gly	GAT Asp	ATA Ile	TCT Ser 1670	Ser	ACT Thr	GTT Val	ATC Ile	AAT Asn 167	TTC Phe 5,	TCT Ser	CAA Gln	AAG Lys	TAT Tyr 1680	5040
35	CTT Leu	TAT Tyr	GGA Gly	ATA Ile	GAC Asp 1689	Ser	TGT Cys	GTT Val	AAT Asn	AAA Lys 1690	Val	GTA Val	ATT Ile	TCA Ser	CCA Pro 1699	Asn	5088
40	ATT Ile	TAT Tyr	ACA Thr	GAT Asp 1700	GIU	ATA Ile	AAT Asn	ATA Ile	ACG Thr 1705	Pro	GTA Val	TAT Tyr	GAA Glu	ACA Thr 1710	Asn	AAT Asn	5136
	ACT Thr	TAT Tyr	CCA Pro 1715	GIU	GTT Val	ATT Ile	GTA Val	TTA Leu 1720	Asp	GCA Ala	AAT Asn	TAT Tyr	ATA Ile 1725	Asn	GAA Glu	AAA Lys	5184
45	ATA Ile	AAT Asn 1730	vai	AAT Asn	ATC Ile	AAT Asn	GAT Asp 1735	Leu	TCT Ser	ATA Ile	CGA Arg	TAT Tyr 1740	Val	TGG Trp	AGT Ser	AAT Asn	5232
50	GAT Asp 1745	Gry	AAT Asn	GAT Asp	TTT Phe	ATT Ile 1750	Leu	ATG Met	TCA Ser	Thr	AGT Ser 1755	GAA Glu	Glu	Asn	Lys	GTG Val 1760	5280
55	TCA Ser	CAA Gln	GTT Val	AAA Lys	ATA Ile 1765	Arg	TTC Phe	GTT Val	AAT Asn	GTT Val 1770	Phe	AAA Lys	GAT Asp	Lys	ACT Thr 1775	TTG Leu	5328
60	GCA Ala	TAA Asn	AAG Lys	CTA Leu 1780	ser	TTT Phe	AAC Asn	Phe	AGT Ser 1785	Asp	AAA Lys	CAA (Gln .	Asp	GTA Val 1790	Pro	GTA Val	5376
	AGT Ser	GAA Glu	ATA Ile 1795	TTE	TTA Leu	TCA Ser	Phe '	ACA Thr 1800	Pro	TCA Ser	TAT Tyr	TAT (GAG Glu 1805	GAT (Asp (GGA :	TTG Leu	5424
65	ATT Ile	GGC Gly 1810	IYL	GAT Asp	TTG Leu	GIY .	CTA (Leu 1815	GTT Val	TCT Ser	TTA ' Leu '	Tyr	AAT (Asn (1820	GAG . Glu	AAA ' Lys :	TTT ' Phe '	TAT Tyr	5472

	ATT Ile 1825	ASI	AAC Asn	TTT Phe	GGA Gly	ATG Met 183	Met	GTA Val	TCT Ser	GGA Gly	TTA Leu 183	Ile	TAT Tyr	ATT Ile	AAT Asn	GAT Asp 1840	5520
5	TCA Ser	TTA Leu	TAT Tyr	TAT Tyr	TTT Phe 1849	Lys	CCA Pro	CCA Pro	GTA Val	AAT Asn 1850	Asn	TTG Leu	ATA Ile	ACT Thr	GGA Gly 185	Phe	5568
10	GTG Val	ACT Thr	GTA Val	GGC Gly 1860	Asp	GAT Asp	AAA Lys	TAC Tyr	TAC Tyr 186	Phe	AAT Asn	CCA Pro	ATT Ile	AAT Asn 187	Gly	GGA Gly	5616
15	GCT Ala	GCT Ala	TCA Ser 1875	He	GGA Gly	GAG Glu	ACA Thr	ATA Ile 1880	Ile	GAT Asp	GAC Asp	AAA Lys	AAT Asn 1889	Tyr	TAT Tyr	TTC Phe	5664
20	AAC Asn	CAA Gln 1890	ser	GGA Gly	GTG Val	TTA Leu	CAA Gln 1899	Thr	GGT Gly	GTA Val	TTT Phe	AGT Ser 1900	Thr	GAA Glu	GAT Asp	GGA Gly	5712
	TTT Phe 1905	rys	TAT Tyr	TTT Phe	GCC Ala	CCA Pro 1910	Ala	AAT Asn	ACA Thr	CTT Leu	GAT Asp 1915	Glu	AAC Asn	CTA Leu	GAA Glu	GGA Gly 1920	5760
25	GAA Glu	GCA Ala	ATT Ile	GAT Asp	TTT Phe 1925	Thr	GGA Gly	AAA Lys	TTA Leu	ATT Ile 1930	Ile	GAC Asp	GAA Glu	AAT Asn	ATT Ile 1935	Tyr	5808
30	TAT	TTT Phe	GAT Asp	GAT Asp 1940	Asn	TAT Tyr	AGA Arg	GGA Gly	GCT Ala 1949	Val	GAA Glu	TGG Trp	AAA Lys	GAA Glu 1950	Leu	GAT Asp	5856
35	GGT (GLu	ATG Met 1955	His	TAT Tyr	TTT Phe	AGC Ser	CCA Pro 1960	Glu	ACA Thr	GGT Gly	AAA Lys	GCT Ala 1965	Phe	AAA Lys	GGT Gly	5904
40	CTA . Leu	AAT Asn 1970	GIN	ATA Ile	GGT Gly	GAT Asp	TAT Tyr 1975	Lys	TAC Tyr	TAT Tyr	TTC Phe	AAT Asn 1980	Ser	GAT Asp	GGA Gly	GTT Val	5952
	ATG Met 1985	CAA Gln	AAA Lys	GGA Gly	TTT Phe	GTT Val 1990	Ser	ATA Ile	TAA Nsn	GAT Asp	AAT Asn 1995	Lys	CAC His	TAT Tyr	TTT Phe	GAT Asp 2000	6000
45	GAT Asp	TCT Ser	GGT Gly	GTT Val	ATG Met 2005	Lys	GTA Val	GGT Gly	TAC Tyr	ACT Thr 2010	Glu	ATA Ile	GAT Asp	GGC Gly	AAG Lys 2015	His	6048
50	TTC Phe	TAC Tyr	T T T Phe	GCT Ala 2020	Glu	Asn	Gly	Glu	Met	Gln	Ile	GGA Gly	Val	Phe	Asn	ACA Thr	6096
55	GAA (Asp	GGA Gly 2035	Phe	AAA Lys	TAT Tyr	TTT Phe	GCT Ala 2040	His	CAT His	AAT Asn	GAA Glu	GAT Asp 2045	Leu	GGA Gly	AAT Asn	6144
60	GAA (GAA Glu 2050	Gly	GAA Glu	GAA Glu	ATC Ile	TCA Ser 2055	Tyr	TCT Ser	GGT Gly	ATA Ile	TTA Leu 2060	Asn	TTC Phe	AAT Asn	AAT Asn	6192
÷	AAA Lys 2065	ATT Ile	TAC Tyr	TAT Tyr	TTT Phe	GAT Asp 2070	Asp	TCA Ser	TTT Phe	ACA Thr	GCT Ala 2075	Val	GTT Val	GGA Gly	TGG Trp	AAA Lys 2080	6240
65	GAT Asp	TTA Leu	GAG Glu	GAT Asp	GGT Gly 2085	Ser	AAG Lys	TAT Tyr	TAT Tyr	TTT Phe 2090	Asp	GAA Glu	GAT Asp	ACA Thr	GCA Ala 2095	Glu	6288

	GCA Ala	TAT	TATA	GGT Gly 210	Leu	TCA Ser	TTA Leu	ATA Ile	AAT Asn 210	Asp	GGT Gly	CAA Gln	TAT Tyr	TAT Tyr 211	Phe	AAT Asn	6336
5	Asp	Asp	211	Ile 5	Met	Gln	Val	Gly 212	Phe 0	Val	Thr	Ile	Asn 212	Asp 5	Lys		6384
10	Pne	1yr 213	o Phe	Ser	Asp	TCT Ser	Gly 213	Ile 5	Ile	Glu	Ser	Gly 214	Val 0	Gln	Asn	Ile	6432
15	214	Asp 5	Asn	Tyr	Phe	TAT Tyr 215	Ile	Asp	Asp	Asn	Gly 215	Ile 5	Val	Gln	Ile	Gly 2160	6480
20	vai	Pne	Asp	Thr	Ser 216		Gly	Tyr	Lys	Tyr 217	Phe	Ala	Pro	Ala	Asn 217	Thr 5	6528
25	vai	Asn	Asp	Asn 218	Ile O	TAC	Gly	Gln	Ala 218	Val 5	Glu	Tyr	Ser	Gly 2190	Leu)	Val	6576
23	Arg	vai	G1y 219	Glu 5	Asp	GTA Val	Tyr	Tyr 2200	Phe)	Gly	Glu	Thr	Tyr 2205	Thr	Ile	Glu	6624
30	inr	221	Trp 0	Ile	Tyr	GAT Asp	Met 2215	Glu 5	Asn	Glu	Ser	Asp 2220	Lys)	Tyr	Tyr	Phe	6672
35	2225	Pro	Glu	Thr	Lys	AAA Lys 2230	Ala	Cys	Lys	Gly	11e 2239	Asn	Leu	Ile	Asp	Asp 2240	6720
40	IIe	Lys	Tyr	Tyr	Phe 2245		Glu	Lys	Gly	Ile 2250	Met	Arg	Thr	Gly	Leu 2255	Ile	6768
15	ser	Pne	Glu	2260	Asn)	AAT Asn	Tyr	Tyr	Phe 2265	Asn	Glu	Asn	Gly	Glu 2270	Met)	Gln	6816
45	Phe	GΙΫ	туr 2275	lle	Asn	ATA Ile	Glu	Asp 2280	Lys)	Met	Phe	Tyr	Phe 2285	Gly	Glu	Asp	6864
50	GIY	2290	Met)	Gin	Ile	GGA Gly	Val 2295	Phe	Asn	Thr	Pro	Asp 2300	Gly	Phe	Lys	Tyr	6912
55	2305	Ala	HIS	Gin	Asn	ACT Thr 2310	Leu	Asp	Glu	Asn	Phe 2315	Glu	Gly	Glu	Ser	Ile 2320	6960
60	ASN	Tyr	Tnr	GIA	Trp 2325		Asp	Leu	Asp	Glu 2330	Lys	Arg	Tyr	Tyr	Phe 2335	Thr	7008
, -	Asp	Glu	Tyr	11e 2340	Ala	GCA Ala	Thr	Gly	Ser 2345	Val	Ile	Ile	Asp	Gly 2350	Glu	GAG Glu	7056
65	TAT	TAT Tyr	TTT Phe 2355	Asp	CCT Pro	GAT Asp	Thr	GCT Ala 2360	Gln	TTA Leu	GTG Val	Ile	AGT Ser 2365	GAA Glu			7098
70	TAG																7101

(2) INFORMATION FOR SEQ ID NO:10:

5	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 2366 amino acids (B) TYPE: amino acid															
				(D) TO	POLO	GY:	line	ar							
10			•	MOLE												
117	Mor			SEQU												
	•				5					10					15	
15	Phe	Arg	Thr	Gln 20	Glu	Asp	Glu	Tyr	Val 25	Ala	Ile	Leu	Asp	Ala 30		Glu
20	Glu	Tyr	His 35	Asn	Met	Ser	Glu	Asn 40	Thr	Val	Val	Glu	Lys 45	Tyr	Leu	Lys
	Leu	Lys 50	Asp	Ile	Asn	Ser	Leu 55	Thr	Asp	Ile	Tyr	Ile 60	Asp	Thr	Tyr	Lys
25	Lys 65	Ser	Gly	Arg	Asn	Lys 70	Ala	Leu	Lys	Lys	Phe 75	Lys	Glu	Tyr	Leu	Val 80
<u>.</u>	Thr	Glu	Val	Leu	Glu 85	Leu	Lys	Asn	Asn	Asn 90	Leu	Thr	Pro	Val	Glu 95	Lys
30	Asn	Leu	His	Phe 100	Val	Trp	Ile	Gly	Gly 105	Gln	Ile	Asn	Asp	Thr 110	Ala	Ile
35	Asn	Tyr	Ile 115	Asn	Gln	Trp	Lys	Asp 120	Val	Asn	Ser	Asp	Tyr 125	Asn	Val	Asn
	Val	Phe '130	Tyr	Asp	Ser	Asn	Ala 135	Phe	Leu	Ile	Asn	Thr 140	Leu	Lys	Lys	Thr
40	Val 145	Val	Glu	Ser	Ala	Ile 150	Asn	Asp	Thr	Leu	Glu 155	Ser	Phe	Arg	Glu	Asn 160
	Leu	Asn	Asp	Pro	Arg 165	Phe	Asp	Tyr	Asn	Lys 170	Phe	Phe	Arg	Lys	Arg 175	Met
45	Glu	Ile	Ile	Tyr 180	Asp	Lys	Gln	Lys	Asn 185	Phe	Ile	Asn	туг	Tyr 190	Lys	Ala
50	Gln	Arg	Glu 195	Glu	Asn	Pro	Glu	Leu 200	Ile	Ile	Asp	Asp	Ile 205	Val	Lys	Thr
	Tyr	Leu 210	Ser	Asn	Glu	Tyr	Ser 215	Lys	Glu	Ile	Asp	Glu 220	Leu	Asn	Thr	Tyr
55	Ile 225	Glu	Glu	Ser	Leu	Asn 230	Lys	Ile	Thr	Gln	Asn 235	Ser	Gly	Asn	Asp	Val 240
	Arg	Asn	Phe	Glu	Glu 245	Phe	Lys	Asn	Gly	Glu 250	Ser	Phe	Asn	Leu	Tyr 255	Glu
60	Gln	Glu	Leu	Val 260	Glu	Arg	Trp	Asn	Leu 265	Ala	Ala	Ala	Ser	Asp 270	Ile	Leu
65	Arg	Ile	Ser 275	Ala	Leu	Lys	Glu	11e 280	Gly	Gly	Met	Tyr	Leu 285	Asp	Val	Asp
	Met	Leu 290	Pro	Gly	Ile	Gln	Pro 295	Asp	Leu	Phe	Glu	Ser 300	Ile	Glu	Lys	Pro
70	Ser 305	Ser	Val	Thr	Val	Asp 310	Phe	Trp	Glu	Met	Thr 315	Lys	Leu	Glu	Ala	Ile 320

ı	Met	Lys	Tyr	Lys	Glu 325	Tyr	Ile	Pro	Glu	Tyr 330		Ser	Glu	His	Phe	Asp
5	Met	Leu	Asp	Glu 340	Glu	Val	Gln	Ser	Ser 345		Glu	Ser	Val	Leu 350		Ser
	Lys	Ser	Asp 355	Lys	Ser	Glu	Ile	Phe 360	Ser	Ser	Leu	Gly	Asp 365		Glu	Ala
10	Ser	Pro 370	Leu	Glu	Val	Lys	Ile 375	Ala	Phe	Asn	Ser	Lys 380		Ile	Ile	Asn
15	Gln 385	Gly	Leu	Ile	Ser	Val 390	Lys	Asp	Ser	Tyr	Cys 395	Ser	Asn	Leu	Ile	Val 400
	Lys	Gln	Ile	Glu	Asn 405	Arg	Tyr	Lys	Ile	Leu 410	Asn	Asn	Ser	Leu	Asn 415	Pro
20	Ala	Ile	Ser	Glu 420	Asp	Asn	Asp	Phe	Asn 425	Thr	Thr	Thr	Asn	Thr 430	Phe	Ile
	Asp	Ser	Ile 435	Met	Ala	Glu	Ala	Asn 440	Ala	Asp	Asn	Gly	Arg 445	Phe	Met	Met
25	Glu	Leu 450	Gly	Lys	Tyr	Leu	Arg 455	Val	Gly	Phe	Phe	Pro 460	Asp	Val	Lys	Thr
30	Thr 465	Ile	Asn	Leu	Ser	Gly 470	Pro	Glu	Ala	Tyr	Ala 475	Ala	Ala	Tyr	Gln	Asp 480
	Leu	Leu	Met	Phe	Lys 485	Glu	Gly	Ser	Met	Asn 490	Ile	His	Leu	Ile	Glu 495	Ala
35	Asp	Leu	Arg	Asn 500	Phe	Glu	Ile	Ser	Lys 505	Thr	Asn	Ile	Ser	Gln 510	Ser	Thr
	Glu	Gln	Glu 515	Met	Ala	Ser	Leu	Trp 520	Ser	Phe	Asp	Asp	Ala 525	Arg	Ala	Lys
40		530			Glu		535					540				_
45	545				Leu	550					555				-	560
ı					Lys 565					570					575	_
50				580	Ile				585					590		
			595		Leu			600					605			
55		610			Glu		615					620				
60	Asp 625	Gly	Glu	Ile	Gln	Glu 630	Ile	Asp	Lys	Tyr	Lys 635	Ile	Pro	Ser	Ile	Ile 640
					Lys 645					650					655	
65				660	Asp				665					670		
	Thr	Glu	Ile 675	Glu	Ala	Ala	Ile	Asp 680	Leu	Ala	Lys	Glu	Asp 685	Ile	Ser	Pro

	Lys	Ser 690	lle	Glu	Ile	Asn	Leu 695	Leu	Gly	/ Cys	Asn	Met 700	Phe	Ser	Tyr	Ser
5	Ile 705	Asn	Val	Glu	Glu	Thr 710	Tyr	Pro	Gly	/ Lys	Leu 715	Leu	Leu	Lys	. Val	Lys 720
	Asp	Lys	Ile	Ser	Glu 725	Leu	Met	Pro	Ser	1le 730	Ser	Gln	Asp	Ser	1le 735	
10	Val	Ser	Ala	Asn 740	Gln	Tyr	Glu	Val	Arg 745	Ile	Asn	Ser	Glu	Gly 750		Arg
15	Glu	Leu	Leu 755	Asp	His	Ser	Gly	Glu 760	Trp	Ile	Asn	Lys	Glu 765	Glu	Ser	Ile
	Ile	Lys 770	Asp	Ile	Ser	Ser	Lys 775	Glu	Туr	Ile	Ser	Phe 780	Asn	Pro	Lys	Glu
20	Asn 785	Lys	Ile	Thr	Val	Lys 790	Ser	Lys	Asn	Leu	Pro 795	Glu	Leu	Ser	Thr	Leu 800
	Leu	Gln	Glu	Ile	Arg 805	Asn	Asn	Ser	Asn	Ser 810	Ser	Asp	Ile	Glu	Leu 815	Glu
25			Val	020					825					830		
30	Asp	Thr	Gin 835	Ile	Val	Glu	Glu	Arg 840	Ile	Glu	Glu	Ala	Lys 845	Asn	Leu	Thr
		050	Ser				855					860				
35	003		Asp			870					875					880
40			Phe		000					890					895	
40			Arg	900					905					910		
45			Lys 915					920					925			
		230	Lys				935					940				
50	743		Lys			950					955					960
			Phe		202					970					975	
55			Ser	980					985					990		
50			Ser 995					1000)				1005			
		1010					1015) i				1020				
55	1025	,	Glu			1030					1035	•				1040
	Leu	Gly	Ala	Ala	Ile 1045	Lys	Glu	Leu	Ser	Glu 1050	Thr	Ser	Asp		Leu 1055	

	Arg Gln Glu	Ile Glu A 1060	la Lys Ile	e Gly Ile Me 1065	t Ala Va	l Asn Leu 1070	Thr
5	Thr Ala Thr 107	Thr Ala I 5	le Ile Thi	r Ser Ser Le 30	u Gly Ile 108	e Ala Ser 35	Gly
	Phe Ser Ile 1090	Leu Leu V	al Pro Leu 1095	Ala Gly Il	e Ser Ala 1100	a Gly Ile	Pro
10	Ser Leu Val 1105	Asn Asn G	lu Leu Val 110	. Leu Arg As	p Lys Ala 15	Thr Lys	Val 1120
15	Val Asp Tyr	Phe Lys H 1125	is Val Ser	Leu Val Gl	u Thr Glu	Gly Val	
	Thr Leu Leu	Asp Asp L	ys Ile Met	Met Pro Gli 1145	n Asp Asp	Leu Val	Ile
20	Ser Glu Ile 115	Asp Phe As	sn Asn Asn 116	Ser Ile Vai	Leu Gly 116	Lys Cys	Glu
	Ile Trp Arg 1170	Met Glu G	ly Gly Ser 1175	Gly His Th	Val Thr	Asp Asp	Ile
25	Asp His Phe	Phe Ser Al	a Pro Ser 190	Ile Thr Tyr	Arg Glu	Pro His	Leu 1200
30	Ser Ile Tyr	Asp Val Le 1205	eu Glu Val	Glm Lys Glu 1210	ı Glu Leu	Asp Leu 1215	Ser
	Lys Asp Leu	Met Val Le 1220	u Pro Asn	Ala Pro Asn 1225	Arg Val	Phe Ala	Trp
35	Glu Thr Gly 1235	Trp Thr Pr	o Gly Leu 1240	Arg Ser Leu	Glu Asn 124	Asp Gly	Thr
	Lys Leu Leu 1250	Asp Arg Il	e Arg Asp 1255	Asn Tyr Glu	Gly Glu 1260	Phe Tyr	Trp
40	Arg Tyr Phe 1265	Ala Phe Il 12	e Ala Asp 70	Ala Leu Ile 127	Thr Thr		Pro 1280
45	Arg Tyr Glu	Asp Thr As 1285	n Ile Arg	Ile Asn Leu 1290	Asp Ser	Asn Thr #	Arg
	Ser Phe Ile	Val Pro Il 1300	e Ile Thr	Thr Glu Tyr 1305	Ile Arg	Glu Lys I 1310	Leu
50	Ser Tyr Ser		1320	•	1325	i	
	Gln Tyr Asn I 1330	Met Gly Il	e Asn Ile 1335	Glu Leu Ser	Glu Ser 1340	Asp Val 7	rp
55	Ile Ile Asp 1	Val Asp Ası 13!	n Val Val 50	Arg Asp Val 135	Thr Ile		sp 360
60	Lys Ile Lys I	Lys Gly Ası 1365	Leu Ile	Glu Gly Ile 1370	Leu Ser	Thr Leu S 1375	er
	Ile Glu Glu A	Asn Lys Ile 1380	e Ile Leu	Asn Ser His 1385	Glu Ile	Asn Phe S 1390	er
65	Gly Glu Val A	Asn Gly Ser	Asn Gly 1400	Phe Val Ser	Leu Thr	Phe Ser I	le
	Leu Glu Gly I 1410	le Asn Ala	Ile Ile (Glu Val Asp	Leu Leu . 1420	Ser Lys S	er

•	Tyr 142	Lys 5	Leu	Leu	Ile	Ser 1430	Gly	Glu	Leu	Lys	Ile 143		Met	Leu	Asn	Ser 1440
5	Asn	His	Ile	Gln	Gln 1445	Lys	Ile	Asp	Tyr	Ile 1450	Gly	Phe	Asn	Ser	Glu 1459	
	Gln	Lys	Asn	Ile 1460	Pro	Tyr	Ser	Phe	Val 1465		Ser	Glu	Gly	Lys 147		Asn
10	Gly	Phe	Ile 1479	Asn	Gly	Ser	Thr	Lys 1480	Glu)	Gly	Leu	Phe	Val 148		Glu	Leu
15	Pro	Asp 1490	Val	Val	Leu	Ile	Ser 1495	Lys	Val	Tyr	Met	Asp 1500		Ser	Lys	Pro
	Ser 150	Phe 5	Gly	Tyr	Tyr	Ser 1510	Asn)	Asn	Leu	Lys	Asp 1519	Val	Lys	Val	Ile	Thr 1520
20	rys	Asp	Asn	Val	Asn 1525	Ile	Leu	Thr	Gly	Tyr 1530		Leu	Lys	Asp	Asp 1535	
	Lys	Ile	Ser	Leu 1540	Ser	Leu	Thr	Leu	Gln 1545	Asp	Glu	Lys	Thr	Ile 1550		Leu
25		Ser	1222	•				1560)				1565	6		
30	Met	Asn 1570	Arg	Lys	Gly	Asn	Thr 1575	Asn	Thr	Ser	Asp	Ser 1580		Met	Ser	Phe
	Leu 1585	Glu 5	Ser	Met	Asn	Ile 1590	Lys)	Ser	Ile	Phe	Val 1599		Phe	Leu	Gln	Ser 1600
35		Ile			1605	•				1610)			_	1615	
		Ile		1620)				1625	i				1630)	
40		Tyr	1635	•				1640)				1645	5		
45		Gly 1650)				1655	•				1660)			
•	1665					1670)				1675	5				1680
50		Tyr			1685	•				1690)				1695	i
2.5		Tyr		1700)				1705	•				1710)	
55		Tyr	1715	•				1720)				1725	5		-
60		Asn 1730)				1735	,				1740)			
	1745					1750)				1755	•				1760
65		Gln			1765	i				1770)				1775	•
	Ala	Asn	Lys	Leu 1780	Ser	Phe	Asn	Phe	Ser 1785		Lys	Gln	Asp	Val 1790		Val

	Ser	Glu	11e 179	Ile	Leu	Ser	Phe	Thr 1800		Ser	Tyr	Tyr	Glu 180		Gly	Leu
5	Ile	Gly 181	Tyr	Asp	Leu	Gly	Leu 181		Ser	Leu	Tyr	Asn 182		Lys	Phe	Tyr
	Ile 182	Asn 5	Asn	Phe	Gly	Met 1830		Val	Ser	Gly	Leu 1835		Tyr	lle	Asn	Asp 1840
10	Ser	Leu	Tyr	Tyr	Phe 184	Lys	Pro	Pro	Val	Asn 1850		Leu	Ile	Thr	Gly 185	
15	Val	Thr	Val	Gly 1860	Asp	Asp	Lys	Tyr	Tyr 1865		Asn	Pro	Ile	Asn 1870		Gly
1.7	Ala	Ala	Ser 1879	Ile	Gly	Glu	Thr	Ile 1880		Asp	Asp	Lys	Asn 1889		туr	Phe
20	Asn	Gln 1890	Ser	Gly	Val	Leu	Gln 1895	Thr	Gly	Val	Phe	Ser 1900		Glu	Asp	Gly
	Phe 190	Lys	Tyr	Phe	Ala	Pro 1910	Ala	Asn	Thr	Leu	Asp 1915		Asn	Leu	Glu	Gly 1920
25	Glu	Ala	lle	Asp	Phe 1925	Thr	Gly	Lys	Leu	Ile 1930		Asp	Glu	Asn	Ile 1935	
20	Tyr	Phe	Asp	Asp 1940	Asn	Tyr	Arg	Gly	Ala 1945		Glu	Trp	Lys	Glu 1950		Asp
30	Gly	Glu	Met 1955	His	туr	Phe	Ser	Pro 1960		Thr	Gly	Lys	Ala 1965		Lys	Gly
35	Leu	Asn 1970	Gln	Ile	Gly	Asp	Tyr 1975	Lys	Tyr	Tyr	Phe	Asn 1980		Asp	Gly	Val
	Met 1985	Gln	Lys	Gly	Phe	Val 1990	Ser	Ile	Asn	Asp	Asn 1995		His	Tyr	Phe	Asp 2000
40	Asp	Ser	Gly	Val	Met 2005	Lys	Val	Gly	Tyr	Thr 2010		Ile	qsA	Gly	Lys 2015	
45	Phe	туr	Phe	Ala 2020	Glu	Asn	Gly		Met 2025		Ile	Gly	Val	Phe 2030		Thr
4.1	Glu	Asp	Gly 2035	Phe	Lys	Tyr	Phe	Ala 2040		His	Asn	Glu	Asp 2045		Gly	Asn
50	Glu	Glu 2050	Gly	Glu	Glu	Ile	Ser 2055		Ser	Gly		Leu 2060		Phe	Asn	Asn
	Lys 2065	Ile	Tyr	Tyr	Phe	Asp 2070	Asp	Ser	Phe	Thr	Ala 2075	Val	Val	Gly	Trp	Lys 2080
55	Asp	Leu	Glu	Asp	Gly 2085	Ser	Lys	Tyr	Tyr	Phe 2090	Asp	Glu	Asp	Thr	Ala 2095	
60	Ala	Tyr	Ile	Gly 2100	Leu	Ser	Leu	Ile	Asn 2105	Asp	Gly	Gln	Tyr	Туг 2110		Asn
.,,,	Asp	Asp	Gly 2115	Ile	Met	Gln	Val	Gly 2120		Val	Thr		Asn 2125		Lys	Vai
65	Phe	Tyr 2130	Phe	Ser	Asp	Ser	Gly 2135		Jle	Glu		Gly 2140		Gln	Asn	Ile
	Asp 2145	Asp	Asn	Tyr	Phe	Tyr 2150		Asp	Asp	Asn	Gly 2155		Val	Gln	Ile	Gly 2160
70	Val	Phe	Asp	Thr	Ser	Asp	Gly	Tyr	Lys	Tyr	Phe	Ala	Pro	Ala	Asn	Thr

						216	5				217	0				217	5
5	Va.	1	Asn	Asp	Asn 218	Ile O	Tyr	Gly	Gln	Ala 218	Val 5	Glu	Tyr	Ser	Gly 219	Leu 0	Val
					Glu 5				220	U				220	5		
10					Ile				_				222	U			
					Thr			•				223	5				224
15					Tyr		-				2250	,				225	5
20					Asn 2260					220.	,				2270)	
									2200	,				2289	5		
25					Gln				,				2300)			
30					Gln			•				2315	,				2320
507											2330					2335	
35					Ile 2340					2343					2350	Glu	Glu
•	(2)				Asp ION				2360		Leu	Val	Ile	Ser 2365	Glu		
40 45				SEQ (A (B	UENC:) LEI) TY:) STI	E CHANGTH PE: 1	ARAC : 19 nucl EDNE	TERI bas eic SS:	STIC e pa acid sing	S: irs							
					ECULI												
50					UENCI		SCRI	PTIO	N: S	EQ II	ои о	:11:					
					GCAAJ												
55	(2)			SEQU	ION I	CHA	RAC	reri:	STICS	3 ·							
50				(A) (B) (C)	LEN TYPE STE TOP	NGTH: PE: n RANDE	21 ucle DNE	base eic a	e pa: acid singl	irs							
		(;	ii)	MOLE	CULE	TYP	PE: 1	ANC	(genc	omic)							
		(>	(i)	SEQU	JENCE	DES	CRI	PTIO	1: SE	EQ IE	NO:	12:					
55	TTTC	'A'	CTI	G TA	GAGT	CAAA	G										
	(2)	IN	IFOR	MATI	ON F	OR S	EQ 1	D NO):13:								
7()		(i)	SEQU (A)	JENCE LEN	CHA	RAC1	TERIS base	TICS	: .rs							

	(B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
5	(ii) MOLECULE TYPE: DNA (genomic)	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:	
10	GATGCCACAA GATGATTTAG TG	2:
10	(2) INFORMATION FOR SEQ ID NO:14:	
15	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 22 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
20	(ii) MOLECULE TYPE: DNA (genomic)	
20	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:	
	CTAATTGAGC TGTATCAGGA TC	22
25	(2) INFORMATION FOR SEQ ID NO:15:	
30	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 27 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
	(11) MOLECULE TYPE: DNA (genomic)	
35	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:	
	CGGAATTCCT AGAAAAAATG GCAAATG	27
4.0	(2) INFORMATION FOR SEQ ID NO:16:	
40	(1) SEQUENCE CHARACTERISTICS: (A) LENGTH: 26 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: DNA (genomic)	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:	
50 -	GCTCTAGAAT GACCATAAGC TAGCCA	26
	(2) INFORMATION FOR SEQ ID NO:17:	
55	(i) SEQUENCE CHARACTERISTICS:	
60	(A) LENGTH: 27 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
OO	(ii) MOLECULE TYPE: DNA (genomic)	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:	
65	CGGAATTCGA GTTGGTAGAA AGGTGGA	27
	(2) INFORMATION FOR SEQ ID NO:18:	
70	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 27 base pairs	

27

28

		(C)	TYPE: STRANI TOPOLO	DEDNE	ESS:	sinc	i gle								
. 5	(ii)	MOLE	CULE TY	PE:	DNA	(ger	omic	:)							
	(xi)	SEQU	ENCE DE	SCRI	PTIC	N: S	EQ I	D NO	0:18:						
10	CGGAATTC	GG TT	ATTATCI	T AA	GGAT	'G									
10	(2) INFO	RMATI	ON FOR	SEQ	ID N	O:19	·:								
15	(i)	(A) (B) (C)	ENCE CH LENGTH TYPE: STRAND TOPOLO	l: 28 nucl EDNE	bas eic SS:	e pa acid sing	ırs								
20	(ii)	MOLE	CULE TY	PE:	DNA	(gen	omic)							
	(xi)	SEQUE	ENCE DE	SCRI	PTIO	N: S	EQ I	D NO	:19:						
	CGGAATTC	TT GAT	FAACTGG	А ТТ	TGTG	AC									
25	(2) INFO	RMATIC	ON FOR	SEQ	ID N	0:20	:								
30		SEQUE (A) (B) (C)	ENCE CH LENGTH TYPE: STRAND TOPOLO	ARAC : 51 amin EDNE	TERI 1 am 0 ac SS: 1	STIC ino id unkn	S: acid	s							
	(ii)	MOLEC	ULE TY	PE: ¡	prote	ein									
35	(xi)	SEQUE	NCE DE	SCRI	PTIO	N: SI	EQ I	ои о	:20:						
	Leu 1	Ile T	hr Gly	Phe 5	Val	Thr	Val	Gly	Asp 10	Asp	Lys	Tyr	Tyr	Phe 15	Asn
4()	Pro	Ile A	sn Gly 20	Gly	Ala	Ala	Ser	Ile 25	Gly	Glu	Thr	Ile	Ile 30	Asp	Asp
45	Lys	Asn T	yr Tyr 5	Phe	Asn	Gln	Ser 40	Gly	Val	Leu	Gln	Thr 45	Gly	Val	Phe
	Ser	Thr G 50	lu Asp	Gly	Phe	Lys 55	Tyr	Phe	Ala	Pro	Ala 60	Asn	Thr	Leu	Asp
50	Glu 65	Asn L	eu Glu	Gly	Glu 70	Ala	Ile	Asp	Phe	Thr 75	Gly	Lys	Leu	Ile	Ile 80
	Asp	Glu A	sn Ile	Tyr 85	Tyr	Phe	Asp	Asp	Asn 90	Tyr	Arg	Gly	Ala	Val 95	Glu
55	Trp	Lys G	lu Leu 100	Asp	Gly	Glu	Met	His 105	Tyr	Phe	Ser	Pro	Glu 110	Thr	Gly
60	Lys	Ala P	he Lys 15	Gly	Leu	Asn	Gln 120	Ile	Gly	Asp	Tyr	Lys 125		Tyr	Phe
. M	Asn	Ser A 130	sp Gly	Val	Met	Gln 135	Lys	Gly	Phe	Val	Ser 140		Asn	Asp	Asn
65	Lys 145	His T	yr Phe	Asp	Asp 150	Ser	Gly	Val	Met	Lys 155	Val	Gly	Tyr	Thr	Glu 160
	Ile	Asp G	ly Lys	His 165	Phe	Tyr	Phe	Ala	Glu 170	Asn	Gly	Glu	Met	Gln 175	Ile
7()	Gly	Val P	he Asn	Thr	Glu	Asp	Gly	Phe	Lys	Tyr	Phe	Ala	His	His	Asn

					180					185					190		
5		Glu	Asp	Leu 195	Gly	Asn	Glu	Glu	Gly 200	Glu	Glu	Ile	Ser	Tyr 205		Gly	Ile
•'		Leu	Asn 210	Phe	Asn	Asn	Lys	Ile 215	Tyr	Туr	Phe	Asp	Asp 220		Phe	Thr	Ala
10		Val 225	Val	Gly	Trp	Lys	Asp 230	Leu	Glu	Asp	Gly	Ser 235	Lys	Туr	Tyr	Phe	Asp 240
		Glu	Asp	Thr	Ala	Glu 245	Ala	Tyr	Ile	Gly	Leu 250	Ser	Leu	Ile	Asn	Asp 255	Gly
15		Gln	Tyr	Tyr	Phe 260	Asn	Asp	Asp	Gly	Ile 265	Met	Gln	Val	Gly	Phe 270	Val	Thr
20		Ile	Asn	Asp 275	Lys	Val	Phe	Tyr	Phe 280	Ser	Asp	Ser	Gly	Ile 285	Ile	Glu	Ser
		Gly	Val 290	Gln	Asn	Ile	Asp	Asp 295	Asn	Tyr	Phe	Tyr	Ile 300	Asp	Asp	Asn	Gly
25		Ile 305	Val	Gln	Ile	Gly	Val 310	Phe	Λsp	Thr	Ser	Asp 315	Gly	туг	Lys	Tyr	Phe 320
		Ala	Pro	Ala	Asn	Thr 325	Val	Asn	Asp	Asn	Ile 330	Tyr	Gly	Gln	Ala	Val 335	Glu
30		Tyr	Ser	Gly	Leu 340	Val	Arg	Val	Gly	Glu 345	Asp	Val	Tyr	Tyr	Phe 350	Gly	Glu
35		Thr	Tyr	Thr 355	Ile	Glu	Thr	Gly	Trp 360	Ile	Tyr	Asp	Met	Glu 365	Asn	Glu	Ser
	•	Asp	Lys 370	Tyr	Tyr	Phe	Asn	Pro 375	Glu	Thr	Lys	Lys	Ala 380	Cys	Lys	Cly	lle
40		Asn 385	Leu	Ile	Asp	Asp	Ile 390	Lys	Tyr	Tyr	Phe	Asp 395	Glu	Lys	Gly	Ile	Met 400
		Arg	Thr	Gly	Leu	Ile 405	Ser	Phe	Glu	Asn	Asn 410	Asn	Tyr	Tyr	Phe	Asn 415	Glu
45		Asn	Gly	Glu	Met 420	Gln	Phe	Gly	Tyr	Ile 425	Asn	Ile	Glu	Asp	Lys 430	Met	Phe
50		Tyr	Phe	Gly 435	Glu	Asp	Gly	Val	Met 440	Gln	Ile	Gly	Val	Phe 445	Asn	Thr	Pro
		Asp	Gly 450	Phe	Lys	Tyr	Phe	Ala 455	His	Gln	Asn	Thr	Leu 460	Asp	Glu	Asn	Phe
55		Glu 465	Gly	Glu	Ser	Ile	Asn 470	Tyr	Thr	Gly	Trp	Leu 475	Asp	Leu	Asp	Glu	Lys 480
		Arg	Tyr	Tyr	Phe	Thr 485	Asp	Glu	Tyr	Ile	Ala 490	Ala	Thr	Gly	Ser	Val 495	Ile
60		Ile	Asp	Gly	Glu 500	Glu	Tyr	Tyr	Phe	Asp 505	Pro	Asp	Thr	Ala	Gln 510	Leu	

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:																
5	(i)	(A) (B) (C)	LEN TYI STI	NGTH PE: 8 RANDI	ARACT 608 amino EDNES GY: V	am: ac: ss: u	ino a id unkno	acids	5							
10	(ii)	MOLE	CULE	TY!	PE: 1	prote	in									
•	(xi)	SEQU	JENCE	DES	CRII	OIT	≬: SE	EQ II	NO:	21:						
15	Ser 1	Glu	Glu	Asn	Lys 5	Val	Ser	Gln	Val	Lys 10	Ile	Arg	Phe	Val	Asn 15	Val
	Phe	Lys	Asp	Lys 20	Thr	Leu	Ala	Asn	Lys 25	Leu	Ser	Phe	Asn	Phe 30	Ser	Asp
20	Lys	Gln	Asp 35	Val	Pro	Val	Ser	Glu 40	Ile	Ile	Leu	Ser	Phe 45	Thr	Pro	Ser
	Tyr	Туr 50	Glu	Asp	Gly	Leu	Ile 55	Gly	Tyr	Asp	Leu	Gly 60	Leu	Val	Ser	Leu
25	Tyr 65	Asn	Glu	Lys	Phe	Tyr 70	Ile	Asn	Asn	Phe	Gly 75	Met	Met	Val	Ser	Gly 80
30	Leu	lìe	Tyr	Ile	Asn 85	Asp	Ser	Leu	Tyr	Tyr 90	Phe	Lys	Pro	Pro	Val 95	Asn
	Asn	Leu	Ile	Thr 100	Gly	Phe	Val	Thr	Val 105	Gly	Asp	Asp	Lys	Tyr 110	Tyr	Phe
35	Asn	Pro	Ile 115	Asn	Gly	Gly	Ala	Ala 120	Ser	Ile	Gly	Glu	Thr 125	Ile	Ile	Asp
	Asp	Lys 130	Asn	Tyr	Tyr	Phe	Asn 135	Gln	Ser	Gly	Val	Leu 140	Gln	Thr	Gly	Val
4()	145	Ser				150					155					160
45	Asp	Glu	Asn	Leu	Glu 165	Gly	Glu	Ala	Ile	Asp 170	Phe	Thr	Gly	Lys	Leu 175	Ile
	Ile	Asp	Glu	Asn 180	Ile	Tyr	Tyr	Phe	Asp 185	Asp	Asn	Tyr	Arg	Gly 190	Ala	Val
5θ	Glu	Trp	Lys 195	Glu	Leu	Asp	Gly	Glu 200	Met	His	Tyr	Phe	Ser 205	Pro	Glu	Thr
		Lys 210		Phe	Lys	Gly	Leu 215	Asn	Gln			Asp 220		Lys	Tyr	Tyr
55	Phe 225	Asn	Ser	Asp	Gly	Val 230	Met	Gln	Lys	Gly	Phe 235	Val	Ser	Ile	Asn	Asp 240
60	Asn	Lys	His	Tyr	Phe 245	Asp	Asp	Ser	Gly	Val 250	Met	Lys	Val	Gly	Tyr 255	Thr
	Glu	Ile	Asp	Gly 260	Lys	His	Phe	Tyr	Phe 265	Ala	Glu	Asn	Gly	Glu 270	Met	Gln
65	Ile	Gly	Val 2 7 5	Phe	Asn	Thr	Glu	Asp 280	Gly	Phe	lys	Tyr	Phe 285	Ala	His	His
	Asn	Glu 290	Asp	Leu	Gly	Asn	Glu 295	Glu	Gly	Glu	Glu	11e 300	Ser	Tyr	Ser	Gly
70	Ile	Leu	Asn	Phe	Asn	Asn	Lys	Ile	Tyr	Tyr	Phe	Asp	Asp	Ser	Phe	Thr

	30	5				310					315					320
5	Al	a Val	Val	Gly	Trp 325	Lys	Asp	Leu	Glu	Asp 330	Gly	Ser	Lys	Tyr	Tyr 335	Phe
•	Asj	p Glu	Asp	Thr 340	Ala	Glu	Ala	Tyr	11e 345	Gly	Leu	Ser	Leu	Ile 350	Asn	Asp
10	G1·	y Gln	Tyr 355	Tyr	Phe	Asn	Asp	Asp 360	Gly	Ile	Met	Gln	Val 365	Gly	Phe	Val
	Th	7 Ile 370	Asn	Asp	Lys	Val	Phe 375	Tyr	Phe	Ser	Asp	Ser 380	Gly	Ile	Ile	Glu
15	38					390					395					400
20		/ Ile			405					410					415	•
		≥ Ala		420					425					430		
25		ı Tyr	435					440					445			•
30		450					455					460				
.10	465					470					475					480
35		Asn			485					490					495	
		Arg		500					505					510		
40		Asn Tyr	515					520					525		_	
45		530 Asp					535					540				
	545	Glu				550					5 5 5					560
50		Arg			565					570					575	
		: Ile		580					585					590		
55	(2) INFO		595					600	1110	vab	110	ASP	605	Λια	GIII	Leu
60		SEQ((A) (B) (C)	JENCE LEN TYP STR		RACT 133 ucle	ERIS 0 ba ic a S: d	TICS se p cid loubl	airs								
65		MOLE			E: D	NA (geno	mic)								
70	(1X)		MAN	E/KE			14									

- 261 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

												•					
5	ATG Met		CGT Arg	CTG Leu	CTG Leu 5	TCT Ser	ACC Thr	TTC Phe	ACT Thr	GAA Glu 10	Tyr	ATC Ile	Lys	AAC Asn	ATC Ile	ATC	4.8
10	AAT Asn	ACC Thr	TCC Ser	ATC Ile 20	Leu	AAC Asn	CTG Leu	CGC Arg	TAC Tyr 25	Glu	TCC	AAT Asn	CAC His	CTG Leu 30	Ile	GAC Asp	96
•	CTG Leu	TCT Ser	CGC Arg 35	IYL	GCT Ala	TCC Ser	AAA Lys	ATC Ile 40	Asn	ATC Ile	GGT Gly	TCT Ser	AAA Lys 45	Val	AAC Asn	TTC Phe	144
15	GAT Asp	CCG Pro 50	116	GAC Asp	AAG Lys	AAT Asn	CAG Gln 55	ATC Ile	CAG Gln	CTG Leu	TTC Phe	AAT Asn 60	CTG Leu	GAA Glu	TCT Ser	TCC Ser	192
20	AAA Lys 65	ATC Ile	GAA Glu	GTT Val	ATC Ile	CTG Leu 70	AAG Lys	AAT Asn	GCT Ala	ATC Ile	GTA Val 75	TAC Tyr	AAC Asn	TCT Ser	ATG Met	TAC Tyr 80	240
25	GAA Glu	AAC Asn	TTC Phe	TCC Ser	ACC Thr 85	TCC Ser	TTC Phe	TGG Trp	ATC Ile	CGT Arg 90	ATC Ile	CCG Pro	AAA Lys	TAC Tyr	TTC Phe 95	AAC Asn	288
30	TCC Ser	ATC Ile	TCT Ser	CTG Leu 100	AAC Asn	AAT Asn	GAA Glu	TAC Tyr	ACC Thr 105	ATC Ile	ATC Ile	AAC Asn	TGC Cys	ATG Met 110	GAA Glu	AAC Asn	336
2-17	AAT Asn	TCT Ser	GGT Gly 115	TGG Trp	AAA Lys	GTA Val	TCT Ser	CTG Leu 120	AAC Asn	TAC Tyr	GG T Gly	GAA Glu	ATC Ile 125	ATC Ile	TGG Trp	ACT Thr	384
35	CTG Leu	CAG Gln 130	GAC Asp	ACT Thr	CAG Gln	GAA Glu	ATC Ile 135	AAA Lys	CAG Gln	CGT Arg	GTT Val	GTA Val 140	TTC Phe	AAA Lys	TAC Tyr	TCT Ser	432
40	CAG Gln 145	ATG Met	ATC Ile	AAC Asn	ATC Ile	TCT Ser 150	GAC Asp	TAC Tyr	ATC Ile	AAT Asn	CGC Arg 155	TGG Trp	ATC Ile	TTC Phe	GTT Val	ACC Thr 160	480
45	ATC Ile	ACC Thr	AAC Asn	AAT Asn	CGT Arg 165	CTG Leu	AAT Asn	AAC Asn	TCC Ser	AAA Lys 170	ATC Ile	TAC Tyr	ATC Ile	AAC Asn	GGC Gly 175	CGT Arg	528
50	CTG Leu	ATC Ile	GAC Asp	CAG Gln 180	AAA Lys	CCG Pro	ATC Ile	TCC Ser	AAT Asn 185	CTG Leu	GGT Gly	AAC Asn	ATC Ile	CAC His 190	GCT Ala	TCT Ser	576
	AAT Asn	7311	ATC Ile 195	ATG Met	TTC Phe	LVS	CTG Leu	Asp	GGT Gly	TGT Cys	CGT Arg	GAC Asp	ACT Thr 205	CAC His	CGC Arg	TAC Tyr	624
55	ATC Ile	TGG Trp 210	ATC Ile	AAA Lys	TAC Tyr	TTC Phe	AAT Asn 215	CTG Leu	TTC Phe	GAC Asp	AAA Lys	GAA Glu 220	CTG Leu	AAC Asn	GAA Glu	AAA Lys	672
60	GAA Glu 225	ATC Ile	AAA Lys	GAC Asp	CTG Leu	TAC Tyr 230	GAC Asp	AAC Asn	CAG Gln	TCC Ser	AAT Asn 235	TCT Ser	GG T Gly	ATC Ile	CTG Leu	AAA Lys 240	720
65	GAC Asp	TTC Phe	TGG Trp	GGT Gly	GAC Asp 245	TAC Tyr	CTG Leu	CAG Gln	TAC Tyr	GAC Asp 250	AAA Lys	CCG Pro	TAC Tyr	TAC Tyr	ATG Met 255	CTG Leu	768
70	AAT Asn	CTG Leu	TAC Tyr	GAT Asp 260	CCG Pro	AAC Asn	AAA Lys	TAC Tyr	GTT Val 265	GAC Asp	GTC Val	AAC Asn	AAT Asn	GTA Val 270	GGT Gly	ATC Ile	816

- 262 -

	CGC Arg	GGT Gly	TAC Tyr 275	ATG Met	TAC Tyr	CTG Leu	AAA Lys	GGT Gly 280	CCG Pro	CGT Arg	GGT Gly	TCT Ser	GTT Val 285	ATG Met	ACT Thr	ACC Thr	864
5	AAC Asn	ATC Ile 290	TAC Tyr	CTG Leu	AAC Asn	TCT Ser	TCC Ser 295	CTG	TAC Tyr	CGT Arg	GGT Gly	ACC Thr 300	AAA	'TTC Phe	ATC Ile	ATC Ile	912
10	AAG Lys 305	AAA Lys	TAC Tyr	GCG Ala	TCT Ser	GGT Gly 310	AAC Asn	AAG Lys	GAC Asp	AAT Asn	ATC Ile 315	GTT Val	CGC Arg	AAC Asn	AAT Asņ	GAT Asp 320	960
15	CGT Arg	GTA Val	TAC Tyr	ATC Ile	AAT Asn 325	GTT Val	GTA Val	GTT Val	AAG Lys	AAC Asn 330	AAA Lys	GAA Glu	TAC Tyr	CGT Arg	CTG Leu 335	GCT Ala	1008
20	ACC Thr	AAT Asn	GCT Ala	TCT Ser 340	CAG Gln	GCT Ala	GGT Gly	GTA Val	GAA Glu 345	AAG Lys	ATC Ile	TTG Leu	TCT Ser	GCT Ala 350	CTG Leu	GAA Glu	1056
	ATC 11e	CCG Pro	GAC Asp 355	GTT Val	GGT Gly	AAT Asn	CTG Leu	TCT Ser 360	CAG Gln	GTA Val	GTT Val	GTA Val	ATG Met 365	AAA Lys	TCC Ser	AAG Lys	1104
25	AAC Asn	GAC Asp 370	CAG Gln	GGT Gly	ATC Ile	ACT Thr	AAC Asn 375	AAA Lys	TGC Cys	AAA Lys	ATG Met	AAT Asn 380	CTG Leu	CAG Gln	GAC Asp	AAC Asn	1152
30	AAT Asn 385	GGT Gly	AAC Asn	GAT Asp	ATC Ile	GGT Gly 390	TTC Phe	ATC Ile	GGT Gly	TTC Phe	CAC His 395	CAG Gln	TTC Phe	AAC Asn	AAT Asn	ATC Ile 400	1200
35	GCT Ala	AAA Lys	CTG Leu	GTT Val	GCT Ala 405	TCC Ser	AAC Asn	TGG Trp	TAC Tyr	AAT Asn 410	CGT Arg	CAG Gln	ATC Ile	GAA Glu	CGT Arg 415	TCC Ser	1248
4()	TCT	CGC Arg	ACT Thr	CTG Leu 420	GGT Gly	TGC Cys	TCT Ser	TGG Trp	GAG Glu 425	TTC Phe	ATC Ile	CCG Pro	GTT Val	GAT Asp 430	GAC Asp	GGT Gly	1296
•••		GGT Gly					TAAC	CCGC	GA A	LODA	T						1330
45	(2)	INFO	ORMAI	CION	FOR	SEQ	ID N	IO:23	3 :								
50			(i) S i i) N	(A) (B) (D)	LEN TYP TOP	IGTH: PE: a POLOG	438 minc SY: 1	ami aci inea	no a d ir		;						
			(i) 5				-			O ID	NO : 2	23:					
55	Met 1	Ala											Lys	Asn	lle 15	Ile	
60	Asn	Thr	Ser	Ile 20	Leu	Asn	Leu	Arg	Tyr 25	Glu	Ser	Asn	His	Leu 30	Ile	Asp	
	Leu	Ser	Arg 35	Tyr	Ala	Ser	Lys	Ile 40	Asn	Ile	Gly	Ser	Lys 45	Val	Asn	Phe	
65	Asp	Pro 50	Ile	Asp	Lys	Asn	Gln 55	Ile	Gln	Leu	Phe	Asn 60	Leu	Glu	Ser	Ser	
70	Lys 65	Ile	Glu	Val	Ile	Leu 70	Lys	Asn	Ala	Ile	Val 75	Tyr	Asn	Ser	Met	Tyr 80	

	Gli	u Ası	ı Phe	Ser	Th:	Ser	Phe	Tr) Ile	Arg	; Ile	Pro	Lys	з Туі	Ph:	e Asn
. 5									105	,				110)	ı Asn
			_					120	,				125	•) Thr
10												140				Ser
15											122					. Thr 160
										1/0					175	
20									103					190		Ser
2.5			Ile 195					200					205			
25			Ile				213					220				
30			Lys			230					235					240
			Trp							250					255	
35			Tyr						265					270		
40			Tyr 275					260					285			
40			Tyr				293					300				
45			Tyr			320					315					320
,			Tyr		J . J					330					335	
50			Ala	3.0					345					350		
55			Asp 355					360					365			
J.,			Gln				3,3					380				
60			Asn .			330					395					400
	Ala				.05					410					415	
65	Ser			120			Ser '	Trp	Glu : 425	Phe	Ile 1	Pro V		Asp . 430	Asp	Gly
70	Trp		435													
70	(2)	INFO	RMAT:	ION I	FOR :	SEQ	ID N	D:24	:							

5		(i		EQUEN (A) I (B) I (C) S (D) I	ENGT TYPE : TRAN	TH: 2 : ami IDEDN	23 an ino a NESS:	nino cid un)	acio									
		(ii	.) MC	DLECU	ILE I	YPE:	pro	teir	1					,				
10		(xi	.) SE	QUEN	ICE D	ESCF	RIPTI	ON:	SEQ	ID N	10:24	:						
10		Me 1	t Gl	у Ні	s Hi	s Hi 5	s Hi	s Hi	s Hi	s Hi	s Hi		s Hi	s Se	er Se	r Gly	His	
15		11	e Gl	u Gl	у Ar 20		s Me	t Al	.a									
	(2)	INF	ORMA	TION	FOR	SEC	ID	NO: 2	:5 :									
20		(i	(QUEN A) L B) T C) S D) T	ENGT YPE: TRAN	H: 1 nuc DEDN	402 leic ESS:	base aci dou	pai d	rs								
25		(li) MO	LECU	LE T	YPE:	DNA	(ge	nomi	c)								
30		(ix	(ATUR A) N B) L	AME/													
				QUEN														
35	ATG Met 1	GLY	CAT	CAT His	CAT His 5	CAT His	CAT His	CAT His	CAT His	CAT His	CAT His	CAC His	AGC Ser	AGC Ser	GGC Gly 15	CAT His		41
40	ATC Ile	GAA Glu	GGT Gly	CGT Arg 20	CAT His	ATG Met	GCT Ala	AGC Ser	ATG Met 25	GCT Ala	CGT Arg	CTG Leu	CTG Leu	TCT Ser	Thr	TTC Phe		96
40	ACT Thr	GAA Glu	TAC Tyr 35	ATC Ile	AAG Lys	AAC Asn	ATC Ile	ATC Ile 40	Asn	ACC Thr	TCC Ser	ATC Ile	CTG Leu 45	AAC Asn	CTG Leu	CGC Arg		144
45	TAC Tyr	GAA Glu 50	TCC Ser	AAT Asn	CAC His	CTG Leu	ATC Ile 55	GAC Asp	CTG Leu	TCT Ser	CGC Arg	TAC Tyr 60	GCT Ala	TCC Ser	AAA Lys	ATC Ile		192
50	AAC Asn 65	ATC Ile	GGT Gly	TC T Ser	AAA Lys	GTT Val 70	AAC Asn	TTC Phe	GAT Asp	CCG Pro	ATC Ile 75	GAC A sp	AAG Lys	AAT Asn	CAG Gln	ATC Ile 80		240
55	C AG Gln	CTG Leu	TTC Phe	AAT Asn	CTG Leu 85	GAA Glu	TCT Ser	TCC Ser	AAA Lys	ATC Ile 90	GAA Glu	GTT Val	ATC Ile	CTG Leu	AAG Lys 95	AAT Asn		288
60	GCT Ala	ATC Ile	GTA Val	TAC Tyr 100	AAC Asn	TCT Ser	ATG Met	TAC Tyr	GAA Glu 105	AAC Asn	TTC Phe	TCC Ser	ACC Thr	TCC Ser 110	TTC Phe	TGG Trp		336
O.O	ATC Ile	CGT Arg	ATC Ile 115	CCG Pro	AAA Lys	TAC Tyr	TTC Phe	AAC Asn 120	TCC Ser	ATC Ile	TCT Ser	CTG Leu	AAC Asn 125	AAT Asn	GAA Glu	TAC Tyr		384
65	ACC Thr	ATC Ile 130	ATC Ile	AAC Asn	TGC Cys	ATG Met	GAA Glu 135	AAC Asn	AAT Asn	TCT Ser	GGT Gly	TGG Trp 140	AAA Lys	GTA Val	TCT Ser	CTG Leu		432
7()	AAC Asn	TAC Tyr	GGT Gly	GAA Glu	ATC Ile	ATC Ile	TGG Trp	ACT Thr	CTG Leu	CAG Gln	GAC Asp	ACT Thr	CAG Gln	GAA Glu	ATC Ile	AAA Lvs		480

	145					150					155					160	
5	CAG Gln	CGT Arg	GTT Val	GTA Val	TTC Phe 165	AAA Lys	TAC Tyr	TCT Ser	CAG Gln	ATG Met 170	ATC Ile	AAC Asn	ATC Ile	TCT Ser	GAC Asp 175	TAC Tyr	528
01	ATC Ile	AAT Asn	CGC Arg	TGG Trp 180	ATC Ile	TTC Phe	GTT Val	ACC Thr	ATC Ile 185	ACC Thr	AAC Asn	AAT Asn	CGT Arg	CTG Leu 190	AAT Asn	AAC Asn	576
	TCC Ser	AAA Lys	ATC Ile 195	TAC Tyr	ATC Ile	AAC Asn	GGC Gly	CGT Arg 200	CTG Leu	ATC Ile	GAC Asp	CAG Gln	AAA Lys 205	CCG Pro	ATC Ile	TCC Ser	624
15	AAT Asn	CTG Leu 210	GGT Gly	AAC Asn	ATC Ile	CAC His	GCT Ala 215	TCT Ser	AAT Asn	AAC Asn	ATC Ile	ATG Met 220	TTC Phe	AAA Lys	CTG Leu	GAC Asp	672
20	GGT Gly 225	TGT Cys	CGT Arg	GAC Asp	ACT Thr	CAC His 230	CGC Arg	TAC Tyr	ATC Ile	TGG Trp	ATC Ile 235	AAA Lys	TAC Tyr	TTC Phe	AAT Asn	CTG Leu 240	720
25	TTC Phe	GAC Asp	AAA Lys	GAA Glu	CTG Leu 245	AAC Asn	GAA Glu	AAA Lys	GAA Glu	ATC Ile 250	AAA Lys	GAC Asp	CTG Leu	TAC Tyr	GAC Asp 255	AAC Asn	768
30	CAG Gln	TCC Ser	AAT Asn	TCT Ser 260	GGT Gly	ATC Ile	CTG Leu	AAA Lys	GAC Asp 265	TTC Phe	TGG Trp	GGT Gly	GAC Asp	TAC Tyr 270	CTG Leu	CAG Gln	816
	TAC Tyr	GAC Asp	AAA Lys 275	CCG Pro	TAC Tyr	TAC Tyr	ATG Met	CTG Leu 280	AAT Asn	CTG Leu	TAC Tyr	GAT Asp	CCG Pro 285	AAC Asn	AAA Lys	TAC Tyr	864
35	GTT Val	GAC Asp 290	GTC Val	AAC Asn	AAT Asn	GTA Val	GGT Gly 295	ATC Ile	CGC Arg	GGT Gly	TAC Tyr	ATG Met 300	TAC Tyr	CTG Leu	AAA Lys	GGT Gly	912
40	CCG Pro 305	CGT Arg	GGT Gly	TCT Ser	GTT Val	ATG Met 310	ACT Thr	ACC Thr	AAC Asn	ATC Ile	TAC Tyr 315	CTG Leu	AAC Asn	TCT Ser	TCC Ser	CTG Leu 320	960
45	TAC Tyr	CGT Arg	GGT Gly	ACC Thr	AAA Lys 325	TTC Phe	ATC Ile	ATC Ile	AAG Lys	AAA Lys 330	TAC Tyr	GCG Ala	TCT Ser	GGT Gly	AAC Asn 335	AAG Lys	1008
50	Asp	Asn	Ile	GTT Val 340	Arg	Asn	Asn	Asp	Arg 345	Val	Tyr	Ile	Asn	Val 350	Val	Val	1056
	AAG Lys	AAC Asn	AAA Lys 355	GAA Glu	TAC Tyr	CGT Arg	CTG Leu	GCT Ala 360	ACC Thr	AAT Asn	GCT Ala	TCT Ser	CAG Gln 365	GCT Ala	GGT Gly	GTA Val	1104
55	GAA Glu	AAG Lys 370	ATC Ile	TTG Leu	TCT Ser	GCT Ala	CTG Leu 375	GAA Glu	ATC Ile	CCG Pro	GAC Asp	GTT Val 380	GGT Gly	AAT Asn	CTG Leu	TCT Ser	1152
60	CAG Gln 385	GTA Val	GTT Val	GTA Val	ATG Met	AAA Lys 390	TCC Ser	AAG Lys	AAC Asn	GAC Asp	CAG Gln 395	GGT Gly	ATC Ile	ACT Thr	AAC Asn	AAA Lys 400	1200
65	TGC Cys	AAA Lys	ATG Met	AAT Asn	CTG Leu 405	CAG Gln	GAC Asp	AAC Asn	TAA naA	GGT Gly 410	AAC Asn	GAT Asp	ATC Ile	GGT Gly	TTC Phe 415	ATC Ile	1248

•	GGT Gly	TTC Phe	CAC His	CAG Gln 420	TTC Phe	AAC Asn	AAT Asn	ATC Ile	GCT Ala 425	Lys	CTG Leu	GTT Val	GCT Ala	TCC Ser 430	AAC Asn	TGG Trp	1296
, 5	TAC Tyr	AAT Asn	CGT Arg 435	CAG Gln	ATC Ile	GAA Glu	CGT Arg	TCC Ser 440	TCT Ser	CGC Arg	ACT Thr	CTG Leu	GGT Gly 445	TGC ,Cys	TCT Ser	TGG Trp	1344
10	GAG Glu	TTC Phe 450	Ile	CCG Pro	GTT Val	GAT Asp	GAC Asp 455	GGT Gly	TGG Trp	GGT Gly	GAA Glu	CGT Arg 460	Pro	CTG Leu			1386
	TAA	CCCG	GGA .	AAGC'	ГT												1402
15	(2)	INF	ORMA	TION	FOR	SEQ	ID I	NO : 2	б:								
20				(B)) LEI) TYI) TOI	NGTH PE: & POLO	: 46: amino GY:	2 am. D ac: linea	ino a id ar		5						
'		(:	xi) :	SEQUI	ENCE	DESC	CRIP	rion	: SE	O ID	NO : 3	26 :					
25	Met 1			His									Ser	Ser	Gly 15	His	
30	Ile	Glu	Gly	Arg 20	His	Met	Ala	Ser	Met 25	Ala	Arg	Leu	Leu	Ser 30	Thr	Phe	
	Thr	Glu	Tyr 35	Ile	Lys	Asn	Ile	Ile 40	Asn	Thr	Ser	Ile	Leu 45	Asn	Leu	Arg	
35	Tyr	Glu 50	Ser	Asn	His	Leu	Ile 55	Asp	Leu	Ser	Arg	Tyr 60	Ala	Ser	Lys	Ile	
40	Asn 65	Ile	Gly	Ser	Lys	Val 70	Asn	Phe	Asp	Pro	Ile 75	Asp	Lys	Asn	Gln	Ile 80	
	Gln	Leu	Phe	Asn	Leu 85	Glu	Ser	Ser	rys	Ile 90	Glu	Val	Ile	Leu	Lys 95	Asn	
45				Tyr 100					105					110			
	Ile	Arg	Ile 115	Pro	Lys	Tyr	Phe	Asn 120	Ser	Ile	Ser	Leu	Asn 125	Asn	Glu	Tyr	
50		130		Asn			135					140					
55	Asn 145	Tyr	Gly	Glu	Ile	Ile 150	Trp	Thr	Leu	Gln	Asp 155	Thr	Gln	Glu	Ile	Lys 160	
	Gln	Arg	Val	Val	Phe 165	Lys	Tyr	Ser	Gln	Met 170	Ile	Asn	Ile	Ser	Asp 175	туг	
60	Ile	Asn	Arg	Trp 180	Ile	Phe	Val	Thr	Ile 185	Thr	Asn	Asn	Arg	Leu 190	Asn	Asn	

	Ser	Lys	Ile 195	Tyr	Ile	Asn	Gly	Arg 200	Leu	Ile	Asp	Gln	Lys 205	Pro	Ile	Ser
5	Asn	Leu 210	Gly	Asn	Ile	His	Ala 215	Ser	Asn	Asn	Ile	Met 220	Phe	Lys	Leu	Asp
	Gly 225	Cys	Arg	Λsp	Thr	His 230	Arg	Tyr	Ile	Trp	11e 235	Lys	Tyr	Phe	Asn	Leu 240
10	Phe	Asp	Lys	Glu	Leu 245	Asn	Glu	Lys	Glu	11e 250	Lys	Asp	Leu	Tyr	Asp 255	
15	Gln	Ser	Asn	Ser 260	Gly	Ile	Leu	Lys	λsp 265	Phe	Trp	Gly	Asp	Tyr 270	Leu	Gln
	туг	Asp	Lys 275	Pro	Tyr	Tyr	Met	Leu 280	Asn	Leu	Tyr	Asp	Pro 285	Asn	Lys	Tyr
20	Val	Asp 290	Val	Asn	Asn	Val	Gly 295	Ile	Arg	Gly	Tyr	Met 300	туг	Leu	Lys	Gly
	Pro 305	Arg	Gly	Ser	Val	Met 310	Thr	Thr	Asn	Ile	Tyr 315	Leu	Asn	Ser	Ser	Leu 320
25		Λrg			343					330					335	
30		Asn		340					345					350		
		Asn						360					365			
35		Lys 370					3/5					380				
•	303	Val				390					395					400
40		Lys			405					410					415	
45	Gly	Phe	His	Gln 420	Phe	Asn	Asn	Ile	Ala 425	Lys	Leu	Val	Ala	Ser 430	Asn	Trp
	Tyr	Asn	Arg 435	Gln	Ile	Glu	Arg	Ser 440	Ser	Arg	Thr	Leu	Gly 445	Суз	Ser	Trp
50	Glu	Phe 450	Ile	Pro	Val	Asp	Asp 455	Gly	Trp	Gly	Glu	Arg 460	Pro	Leu		
	(2)	INFO	RMAT	ION	FOR	SEQ	ID N	0:27	:							
55		(i)	(A	UENC) LE) TY	ngtii	: 38	91 b	ase	pair	s						
,			(C) ST	RAND	EDNE	SS:	doub	le							
60		(ii)		ECUL					omic)						

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..3888

<u>.</u>						CION:											
5						ESCR								1			
10	Met 1	L GII	ı Pne	e vai	. Asn	Lys	Gln	Phe	: Asn	Tyr 10	Lys	. Vsi	Pro	Va]	Asr 15		48
	GTT Val	GA7 Asp	T ATT	GCT Ala 20	Tyr	ATA Ile	AAA Lys	ATT Ile	CCA Pro 25	Asn	GTA Val	GGZ Gly	A CAA / Gln	ATO Met	Glr	CCA Pro	96
15	GTA Val	AAA Lys	A GCT S Ala 35	Pne	AAA Lys	Ile	CAT His	AAT Asn 40	Lys	ATA Ile	TGG Trp	GTT Val	ATT Ile	Pro	GAA Glu	AGA Arg	144
20	GAT Asp	ACA Thr	Pne	ACA Thr	AAT Asn	CCT Pro	GAA Glu 55	GAA Glu	GGA Gly	GAT Asp	TTA Leu	AAT Asn 60	Pro	CCA Pro	CCA Pro	GAA Glu	192
25	GCA Ala 65	Lys	CAA Gln	GTT Val	CCA Pro	GTT Val 70	TCA Ser	TAT Tyr	TAT Tyr	GAT Asp	TCA Ser 75	ACA Thr	TAT Tyr	TTA Leu	AGT Ser	ACA Thr 80	240
30	GAT Asp	AAT Asn	GAA Glu	AAA Lys	GAT Asp 85	AAT Asn	TAT Tyr	TTA Leu	AAG Lys	GGA Gly 90	GTT Val	ACA Thr	AAA Lys	TTA Leu	TTT Phe 95	GAG Glu	288
• "	AGA Arg	ATT Ile	TAT Tyr	TCA Ser 100	ACT Thr	GAT Asp	CTT Leu	GGA Gly	AGA Arg 105	ATG Met	TTG Leu	TTA Leu	ACA Thr	TCA Ser 110	ATA Ile	GTA Val	336
35	AGG Arg	GGA Gly	ATA Ile 115	CCA Pro	TTT Phe	TGG Trp	GGT Gly	GGA Gly 120	AGT Ser	ACA Thr	ATA Ile	GAT Asp	ACA Thr 125	GAA Glu	TTA Leu	AAA Lys	384
40	G T T Val	ATT Ile 130	GAT Asp	ACT Thr	AAT Asn	TGT Cys	ATT Ile 135	AAT Asn	GTG Val	ATA Ile	CAA Gln	CCA Pro 140	GAT Asp	GGT Gly	AGT Ser	TAT Tyr	432
45	AGA Arg 145	TCA Ser	GAA Glu	GAA Glu	CTT Leu	AAT Asn 150	CTA Leu	GTA Val	ATA Ile	ATA Ile	GGA Gly 155	CCC Pro	TCA Ser	GCT Ala	GAT Asp	ATT Ile 160	480
50	ATA Ile	CAG Gln	TTT Phe	GAA Glu	TGT Cys 165	AAA Lys	AGC Ser	TTT Phe	GGA Gly	CAT His 170	GAA Glu	GTT Val	TTG Leu	AAT Asn	CTT Leu 175	ACG Thr	528
	CGA Arg	AAT Asn	GG T Gly	TAT Tyr 180	GGC Gly	TCT Ser	ACT Thr	CAA Gln	TAC Tyr 185	ATT Ile	AGA Arg	TTT Phe	AGC Ser	CCA Pro 190	GAT Asp	TTT Phe	576
. 55	ACA Thr	TTT Phe	GGT Gly 195	TTT Phe	GAG Glu	GAG Glu	TCA Ser	CTT Leu 200	GAA Glu	GTT Val	GAT Asp	ACA Thr	AAT Asn 205	CCT Pro	CTT Leu	TTA Leu	624
60	GGT Gly	GCA Ala 210	GGC Gly	AAA Lys	TTT Phe	GCT Ala	ACA Thr 215	GAT Asp	CCA Pro	GCA Ala	GTA Val	ACA Thr 220	TTA Leu	GCA Ala	CAT His	GAA Glu	672
65	CTT Leu 225	ATA Ile	CAT His	GCT Ala	GIA	CAT His 230	AGA Arg	TTA Leu	TAT Tyr	GGA Gly	ATA Ile 235	GCA Ala	ATT Ile	AAT Asn	CCA Pro	AAT Asn 240	720
70	AGG Arg	GTT Val	TTT Phe	Lys	GTA Val 245	AAT . Asn	ACT .	AAT Asn	Ala	TAT Tyr 250	TAT Tyr	GAA Glu	ATG Met	Ser	GGG Gly 255	TTA Leu	768

•	GAA Glu	GTA Val	AGC Ser	TTT Phe 260	GAG Glu	GAA Glu	CTT Leu	AGA Arg	ACA Thr 265	TTT Phe	GGG Gly	GGA Gly	CAT His	GAT Asp 270	GCA Ala	AAG Lys	816
. 5	TTT Phe	ATA Ile	GAT Asp 275	AGT Ser	TTA Leu	CAG Gln	GAA Glu	AAC Asn 280	GAA Glu	TTT Phe	CGT Arg	CTA Leu	TAT Tyr 285	TAT	TAT Tyr	AAT Asn	864
10	AAG Lys	TTT Phe 290	AAA Lys	GAT Asp	ATA Ile	GCA Ala	AGT Ser 295	ACA Thr	CTT Leu	AAT Asn	AAA Lys	GCT Ala 300	AAA Lys	TCA Ser	ATA Ile	GTA Val	912
15	GGT Gly 305	ACT Thr	ACT Thr	GCT Ala	TCA Ser	TTA Leu 310	CAG Gln	TAT Tyr	ATG Met	AAA Lys	AAT Asn 315	GTT Val	TTT Phe	AAA Lys	GAG Glu	AAA Lys 320	960
20	TAT Tyr	CTC Leu	CTA Leu	TCT Ser	GAA Glu 325	GAT Asp	ACA Thr	TCT Ser	GGA Gly	AAA Lys 330	TTT Phe	TCG Ser	GTA Val	GAT Asp	AAA Lys 335	TTA Leu	1008
	AAA Lys	TTT Phe	GAT Asp	AAG Lys 340	TTA Leu	TAC Tyr	AAA Lys	ATG Met	TTA Leu 345	ACA Thr	GAG Glu	ATT Ile	TAC Tyr	ACA Thr 350	GAG Glu	GAT Asp	1056
25	AAT Asn	TTT Phe	GTT Val 355	AAG Lys	TTT Phe	TTT Phe	AAA Lys	GTA Val 360	CTT Leu	AAC Asn	AGA Arg	AAA Lys	ACA Thr 365	TAT Tyr	TTG Leu	AAT Asn	1104
30	TTT Phe	GAT Asp 370	AAA Lys	GCC Ala	GTA Val	TTT Phe	AAG Lys 375	ATA Ile	AAT Asn	ATA Ile	GTA Val	CCT Pro 380	AAG Lys	GTA Val	AAT Asn	TAC Tyr	1152
35	385	Ile	Tyr	Asp	Gly	TTT Phe 390	Asn	Leu	Arg	Asn	Thr 395	Asn	Leu	Ala	Ala	Asn 400	1200
4()	TTT Phe	AAT Asn	GGT Gly	CAA Gln	AAT Asn 405	ACA Thr	GAA Glu	ATT Ile	AAT Asn	AAT Asn 410	ATG Met	AAT Asn	TTT Phe	ACT Thr	AAA Lys 415	CTA Leu	1248
	AAA Lys	AAT Asn	TTT Phe	ACT Thr 420	GGA Gly	TTG Leu	TTT Phe	GAA Glu	TTT Phe 425	TAT Tyr	AAG Lys	TTG Leu	CTA Leu	TGT Cys 430	GTA Val	AGA Arg	1296
45	GGG Gly	ATA Ile	ATA Ile 435	ACT Thr	TCT Ser	AAA Lys	ACT Thr	AAA Lys 440	TCA Ser	TTA Leu	GAT Asp	AAA Lys	GGA Gly 445	TAC Tyr	AAT Asn	AAG Lys	1344
50	GCA Ala	TTA Leu 450	AAT Asn	Asp	Leu	TG T Cys	Ile	Lys	Val	Asn	Asn	TGG Trp 460	Asp	TTG Leu	TTT Phe	TTT Phe	1392
55	AGT Ser 465	CCT Pro	TCA Ser	GAA Glu	GAT Asp	AAT Asn 470	TTT Phe	ACT Thr	AAT Asn	GAT A sp	CTA Leu 475	AAT Asn	AAA Lys	GGA Gly	GAA Glu	GAA Glu 480	1440
60	ATT Ile	ACA Thr	TCT Ser	GAT Asp	ACT Thr 485	TAA Asn	ATA Ile	GAA Glu	GCA Ala	GCA Ala 490	GAA Glu	GAA Glu	TAA neA	ATT Ile	AGT Ser 495	TTA Leu	1488
	GAT Asp	TTA Leu	AŤA Ile	CAA Gln 500	CAA Gln	TAT Tyr	TAT Tyr	TTA Leu	ACC Thr 505	TTT Phe	AAT Asn	TTT Phe	GAT Asp	AAT Asn 510	GAA Glu	CCT Pro	1536
65	GAA Glu	AAT Asn	ATT Ile 515	TCA Ser	ATA Ile	GAA Glu	AAT Asn	CTT Leu 520	TCA Ser	AGT Ser	GAC Asp	ATT Ile	ATA Ile 525	G1A GCC	CAA Gln	TTA Leu	1584
70	GAA Glu	CTT Leu	ATG Met	CCT Pro	AAT Asn	ATA Ile	GAA Glu	AGA Arg	TTT Phe	CCT Pro	AAT Asn	GGA Gly	AAA Lys	AAG Lys	TAT Tyr	GAG Glu	1632

	•	53	0				53	5				54	0				
5	TT. Le: 54	u no	T AA p Ly	A TA s Ty	T AC' r Th	T ATO	c Pn	C CA' e Hi:	T TA' s Ty:	r CT	CG: Arg	, Al	T CAI	A GAL	A TT	T GAA e Glu 560	1680
10	CA' Hi	r GG s Gl	T AA y Ly	A TC	T AGG T Arg 56!	3 TTE	r GCT ⊇ Ala	r TT a Le	A ACA	A AAT c Asr 570	ı Şer	CT Val	Γ AAC l Asr	GAZ Glu	A GC. 1 Ala 57	A TTA a Leu 5	1728
	TT)	A AA A As:	r cc n Pr	T AG' Se: 580	Arg	r GT1 y Val	TAT	Thi	A TTT	? Phe	TCT Ser	TCA Ser	A GAC Asp	TAT Tyr 590	· Vai	A AAG L Lys	1776
15	AA! Lys	A GT	T AA' l Ası 59	LLys	A GCT S Ala	ACG Thr	GAG Glu	GCA Ala 600	Ala	ATG Met	TTT Phe	TTA Leu	GGC Gly 605	Trp	GT#	GAA Glu	1824
20		610)	LIYI	. ASP) Phe	615	Asp) Glu	Thr	Ser	Glu 620	Val	Ser	Thr	ACG Thr	1872
25	625	Дуз	, 110	. Ala	, Asp	630	inr	He	lle	He	Pro 635	Tyr	Ile	Gly	Pro	GCT Ala 640	1920
30	Deu	NSI.	. 116	Gly	645	мес	Leu	Tyr	Lys	Asp 650	Asp	Phe	Val	Gly	Ala 655		1968
2.5		1110	Jei	660	AIA	vai	rre	reu	665	Glu	Phe	Ile	Pro	Glu 670	Ile		2016
35	**6	FIC	675	Leu	GIY	inr	Pne	680	Leu	Val	Ser	Tyr	11e 685	Ala	Asn		2064
40		690	1111	vai	CAA Gln	1111	695	Asp	Asn	Ala	Leu	Ser 700	Lys	Arg	Asn	Glu	2112
45	705	· · · p	veb	GIU	GTC Val	710	гÀг	Tyr	Ile	Val	Thr 715	Asn	Trp	Leu	Ala	Lys 720	2160
50			****	GIN	ATT Ile 725	Asp	Leu	116	Arg	730	Lys	Met	Lys	Glu	Ala 735	Leu	2208
55		7311	G111	740	GAA Glu	AIA	Inr	rys	745	Ile	Ile	Asn	Tyr	Gln 750	Tyr	Asn	2256
23		- , ~	755	314	GAA Glu	Giu	Lys	760	Asn	11e	Asn	Phe	765	Ile	Asp	Asp	2304
60		770	JŲ.	цуз	CTT Leu	ASII	775	ser	11e	Asn	Lys .	Ala 780	Met	Ile	Asn	Ile	2352
65	785	275	rc	Deu	AAT Asn	790	cys	ser	Val	Ser	Tyr 795	Leu	Met .	Asn	Ser	Met 800	2400
70	ATC Ile	Pro	TAT	GGT Gly	GTT Val 805	AAA Lys .	CGG Arg	TTA Leu	GIU.	GAT Asp 810	TTT (Phe ,	GAT (Asp .	GCT A	Ser .	CTT Leu 815	AAA Lys	2448

	GAT Asp	GCA Ala	TTA Leu	TTA Leu 820	AAG Lys	TAT Tyr	ATA Ile	TAT Tyr	GAT Asp 825	AAT Asn	AGA Arg	GGA Gly	ACT Thr	TTA Leu 830	ATT Ile	GGT Gly	2496
5	CAA Gln	GTA Val	GAT Asp 835	AGA Arg	TTA Leu	AAA Lys	GAT Asp	AAA Lys 840	GTT Val	AAT Asn	AAT Asn	ACA Thr	CTT Leu 845	AGT Ser	ACA Thr	GAT Asp	2544
10	ATA Ile	CCT Pro 850	TTT Phe	CAG Gln	CTT Leu	TCC Ser	AAA Lys 855	TAC Tyr	GTA Val	GAT Asp	AAT Asn	CAA Gln 860	AGA Arg	TTA Leu	TTA Leu	TCT Ser	2592
15	865	TTT Phe	Thr	Glu	Tyr	11e 870	Lys	Asn	Ile	Ile	Asn 875	Thr	Ser	Ile	Leu	Asn 880	2640
20	Leu	AGA Arg	Tyr	GIU	885	Asn	His	Leu	Ile	Asp 890	Leu	Ser	Arg	Tyr	Ala 895	Ser	2688
	Lys	ATA Ile	Asn	900	GIA	Ser	Lys	Val	Asn 905	Phe	Asp	Pro	Ile	Asp 910	Lys	Asn	2736
25	GIN	ATT Ile	915	Leu	Phe	Asn	Leu	Glu 920	Ser	Ser	Lys	Ile	Glu 925	Val	Ile	Leu	2784
30	Lys	AAT Asn 930	Ala	IIe	Val	Tyr	Asn 935	Ser	Met	Tyr	Glu	Asn 940	Phe	Ser	Thr	Ser	2832
35	945	TGG Trp	Ile	Arg	Ile	950	Lys	Tyr	Phe	Asn	Ser 955	Ile	Ser	Leu	Asn	Asn 960	2880
40	GAA [,] Glu	TAT Tyr	ACA Thr	ATA Ile	ATA Ile 965	TAA Asn	TGT Cys	ATG Met	GAA Glu	AAT Asn 970	AAT Asn	TCA Ser	GGA Gly	TGG Trp	AAA Lys 975	GTA Val	2928
	TCA Ser	CTT Leu	AAT Asn	TAT Tyr 980	GGT Gly	GAA Glu	ATA Ile	ATC Ile	TGG Trp 985	ACT Thr	TTA Leu	CAG Gln	GAT Asp	ACT Thr 990	CAG Gln	GAA Glu	2976
45	ATA Ile	AAA Lys	CAA Gln 995	AGA Arg	GTA Val	GTT Val	TTT Phe	AAA Lys 1000	Tyr	AGT Ser	CAA Gln	ATG Met	ATT Ile 1005	Asn	ATA Ile	TCA Ser	3024
50	GAT Asp	TAT Tyr 1010	Ile	AAC Asn	AGA Arg	TGG Trp	ATT Ile 1015	Phe	Val	Thr	ATC Ile	Thr	Asn	AAT Asn	AGA Arg	TTA Leu	3072
55	AAT Asn 1029	AAC Asn	TCT Ser	AAA Lys	ATT Ile	TAT Tyr 1030	Ile	AAT Asn	GGA Gly	AGA Arg	TTA Leu 1035	Ile	GAT Asp	CAA Gln	AAA Lys	CCA Pro 1040	3120
. 60	ATT	TCA Ser	AAT Asn	TTA Leu	GGT Gly 1045	Asn	ATT Ile	CAT His	GCT Ala	AGT Ser 1050	Asn	AAT Asn	ATA Ile	ATG Met	TTT Phe 1055	Lys	3168
	TTA Leu	GAT Asp	GGT Gly	TGT Cys 1060	Arg	GAT Asp	ACA Thr	CAT His	AGA Arg 1065	Tyr	ATT Ile	TGG Trp	ATA Ile	AAA Lys 1070	Tyr	TTT Phe	3216
65	AAT Asn	CTT Leu	TTT Phe 1075	Asp	AAG Lys	GAA Glu	TTA Leu	AAT Asn 1080	Glu	AAA Lys	GAA Glu	ATC Ile	AAA Lys 1085	Asp	TTA Leu	TAT Tyr	3264
70	GAT Asp	AAT Asn	CAA Gln	TCA Ser	AAT Asn	TCA Ser	GGT Gly	ATT Ile	TTA Leu	AAA Lys	GAC Asp	TTT Phe	TGG Trp	GGT Gly	GAT Asp	TAT Tyr	3312

TTA CAA TAT GAT AAA CCA TAC TAT ATG TTA AAT TTA TAT GAT CCA AAT Leu Gln Tyr Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn AAA TAT GTC GAT GTA AAT AAT GTA GGT ATT AGA GGT TAT ATG TAT CTT Lys Tyr Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu AAA GGG CCT AGA GGT AGC GTA ATG ACT ACA AAC ATT TAT TTA AAT TCA Lys Gly Pro Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser AGT TTG TAT AGG GGG ACA AAA TTT ATT ATA AAA AAA TAT GCT TCT GGA Ser Leu Tyr Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly AAT AAA GAT AAT ATT GTT AGA AAT AAT GAT CGT GTA TAT ATT AAT GTA Asn Lys Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val GTA GTT AAA AAT AAA GAA TAT AGG TTA GCT ACT AAT GCA TCA CAG GCA Val Val Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala GGC GTA GAA AAA ATA CTA AGT GCA TTA GAA ATA CCT GAT GTA GGA AAT Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn CTA AGT CAA GTA GTA ATG AAG TCA AAA AAT GAT CAA GGA ATA ACA Leu Ser Gln Val Val Wet Lys Ser Lys Asn Asp Gln Gly Ile Thr AAT AAA TGC AAA ATG AAT TTA CAA GAT AAT AAT GGG AAT GAT ATA GGC Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly TTT ATA GGA TTT CAT CAG TTT AAT AAT ATA GCT AAA CTA GTA GCA AGT Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser AAT TGG TAT AAT AGA CAA ATA GAA AGA TCT AGT AGG ACT TTG GGT TGC Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys TCA TGG GAA TTT ATT CCT GTA GAT GAT GGA TGG GGA GAA AGG CCA CTG Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu TAA (2) INFORMATION FOR SEQ ID NO:28: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 1296 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear (ii) MOLECULE TYPE: protein (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28: Met Gln Phe Val Asn Lys Gln Phe Asn Tyr Lys Asp Pro Val Asn Gly Val Asp Ile Ala Tyr Ile Lys Ile Pro Asn Val Gly Gln Met Gln Pro Val Lys Ala Phe Lys Ile His Asn Lys Ile Trp Val Ile Pro Glu Arg

	-		3	5				4()				45	5		
5	As	p Th 5	r Pho	e Thi	r Asn	Pro	Glu 55	ı Glu	Gly	/ Asp	Leu	Asn 60	Pro	Pro	Pro	Glu
,	Ala 6	a Ly	s Glı	n Val	Pro	Va]	Ser	тук	Туг	Asp	Ser 75	Thr		Leu	Ser	Thr 80
10	Ası	As:	n Glu	ı Lys	Asp 85	Asn	Tyr	Leu	Lys	Gly 90	/ Val	Thr	Lys	Leu	Phe 95	Glu
	Arg	j Ile	e Tyr	Ser 100	Thr	Asp	Leu	Gly	Arg	Met	Leu	Leu	Thr	Ser	I1e	Val
15	Arg	Gly	/ Ile	Pro	Phe	Trp	Gly	Gly 120	Ser	Thr	Ile	Asp	Thr 125	Glu	Leu	Lys
20	Val	11e	e Asp	Thr	Asn	Cys	Ile 135	Asn	Val	Ile	Gln	Pro	Asp	Gly	Ser	Tyr
	Arg 145	Ser	Glu	Glu	Leu	Asn 150	Leu	Val	Ile	Ile	Gly 155	Pro	Ser	Ala	Asp	Ile 160
25	Ile	Glr	Phe	Glu	Cys 165	Lys	Ser	Phe	Gly	His 170	Glu	Val	Leu	Asn	Leu 175	Thr
	Arg	Asn	Gly	Tyr 180	Gly	Ser	Thr	Gln	Tyr 185	Ile	Arg	Phe	Ser	Pro 190	Asp	Phe
30	Thr	Phe	Gly 195	Phe	Glu	Glu	Ser	Leu 200	Glu	Val	Asp	Thr	Asn 205	Pro	Leu	Leu
35	Gly	Ala 210	Gly	Lys	Phe	Ala	Thr 215	Asp	Pro	Ala	Val	Thr 220	Leu	Ala	His	Glu
	Leu 225	Ile	His	Ala	Gly	His 230	Arg	Leu	Tyr	Gly	Ile 235	Ala	Ile	Asn	Pro	Asn 240
40	Arg	Val	Phe	Lys	Val 245	Asn	Thr	Asn	Ala	Tyr 250	Tyr	Glu	Met	Ser	Gly 255	Leu
	Glu	Val	Ser	Phe 260	Glu	Glu	Leu	Arg	Thr 265	Phe	Gly	Gly	His	Asp 270	Ala	Lys
45	Phe	Ile	Asp 275	Ser	Leu	Gln	Glu	Asn 280	Glu	Phe	Arg	Leu	Tyr 285	Tyr	Tyr	Asn
50					Ile		293					300				
						310					315					320
55					Glu . 325					330					335	
40				3.0	Leu '				345					350		
60					Phe			טסנ					365			
65		•			Val 1		3/5					380				
						390					395					400
70	Phe	Asn	Gly	Gln	Asn :	Thr	Glu	Ile /	Asn .	Asn 410	Met i	Asn I	Phe '		Lys 1 415	Leu

	Lys	a Ası	n Phe	e Th:	r Gly	y Lei	u Phe	e Glu	2 Phe 425	€ Ty:	r Lys	s Le	ı Lev	1 Cys		l Arg	
5	Gly	/ 116	¥ 116 439	e Thi	r Sei	r Lys	s Thi	Lys 440	S Ser	Lei	ı Asp	Lys	Gl ₃		Ası	n Lys	
	Ala	1 Let 450	Asr	n Asp) Let	ı Cys	455	Lys i	Val	Ası	n Asn	1 Trp	Asp	Leu	Phe	Phe	
10	Ser 465	Pro	Sei	Glu	a Asp	470	n Phe	Thr	Asn	Asp	Leu 475	Asr	Lys	Gly	Gli	Glu 480	
15	Ile	Thr	Ser	Asp	Thr 485	Asn	ılle	Glu	Ala	Ala 490	Glu	Glu	Asn	Ile	Ser 495	Leu	
	qaA	Leu	Ile	61n 500	Gln	туг	Tyr	Leu	Thr 505	Phe	: Asn	Phe	Asp	Asn 510		Pro	
20	Glu	Asn	1le 515	Ser	Ile	Glu	Asn	Leu 520	Ser	Ser	Asp	Ile	Ile 525	Gly	Gln	Leu	
		330					235					540				Glu	
25	313					550					Arg 555					560	
30					203					570					575		
	Leu	Asn	Pro	Ser 580	Arg	Val	Tyr	Thr	Phe 585	Phe	Ser	Ser	Asp	Tyr 590	Val	Lys	
35			393					600			Phe		605				
		010					615				Ser	620					
40	023					630					Pro 635					640	
45					045					650	Asp				655		
				860					665		Phe			670			
50			0 / 3					680			Ser		685				
• -		0,50					695				Leu	700					
55						110					Thr 715					720	
50					,23					/30	Lys				735		
				/40					745		Ile			750			
55			755					760			Asn		765			_	
tes.		, , ,					//5					780					
0	Asn	Lys	Phe	Leu	Asn	Gln	Cys	Ser	Val	Ser	Tyr	Leu	Met	Asn	Ser	Met	

	785	•				790)				795					800
5	Ile	Pro	Tyr	Gly	Val 805	Lys	Arg	Leu	Glu	Asp 810	Phe	Asp	Ala	Ser	Leu 815	Lys
•	Asp	Ala	Leu	Leu B20	Lys	Tyr	Ile	Tyr	Asp 825	Asn	Arg	Gly	Thr	Leu 830	Ile	Gly
10	Gln	Val	Asp 835	Arg	Leu	Lys	Asp	Lys 840	Val	Asn	Asn	Thr	Leu 845		Thr	Asp
	Ile	Pro 850	Phe	Gln	Leu	Ser	Lys 855	Tyr	Val	Asp	Asn	Gln 860	Arg	Leu	Leu	Ser
15	Thr 865	Phe	Thr	Glu	Tyr	Ile 870	Lys	Asn	Ile	Ile	Asn 875	Thr	Ser	Ile	Leu	Asn 880
20	Leu	Arg	Tyr	Glu	Ser 885	Asn	His	Leu	Ile	Asp 890	Leu	Ser	Arg	Tyr	Ala 895	Ser
	Lys	Ile	Asn	Ile 900	Gly	Ser	Lys	Val	Asn 905	Phe	Asp	Pro	Ile	Asp 910	Lys	Asn
25			713		Phe			920					925			
20		-50			Val		333					940				
30					Ile	950					955					960
35					Ile 965					970					975	
				760	Gly				985					990		
40			,,,		Val			1000	,				1005	5		
1.5			,		Arg		101;	•				1020)			
45		•			Ile	1030	,				1035					1040
50					Gly 1045					1050)				1055	i
				1000					1003	ı				1070)	
55			10,5	,	Lys			1080					1085	•		
50		1050	,		Asn		1095	•				1100				
,()		,				1110					1115					1120
55					Val 1125					1130)				1135	
				1140					1145	'				1150		
70	ser	Leu	Tyr 1155	Arg	Gly	Thr	Lys	Phe 1160	Ile	Ile	Lys		Tyr		Ser	Gly

•	Asn Lys Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val 1170 1175 1180	
5	Val Val Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala 1185 1190 1195 1200	
	Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn 1205 1210 1215	
10	Leu Ser Gln Val Val Wet Lys Ser Lys Asn Asp Gln Gly Ile Thr 1220 1225 1230	
15	Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly 1235 1240 1245	
	Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser 1250 1255 1260	
20	Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys 1265 1270 1275 1280	
	Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu 1285 1290 1295	
25	(2) INFORMATION FOR SEQ ID NO:29:	
30	(i) SEOUENCE CHARACTERISTICS: (A) LENGTH: 30 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
35	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (%1) SEQUENCE DESCRIPTION: SEQ ID NO:29:</pre>	
	CGCCATGGCT AGATTATTAT CTACATTTAC	30
40	(2) INFORMATION FOR SEQ ID NO:30:	30
45	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 26 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
•	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA"</pre>	
50	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:	
	GCAAGCTTCT TGACAGACTC ATGTAG	
55	(2) INFORMATION FOR SEQ ID NO:31:	26
60	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 1546 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: double (D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: DNA (genomic)	
65	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:	
	AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA	60
70	TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACCATG GGCCATCATC	120

	ATCATCATCA TCATCATCAT CACAGCAGCG GCCATATCGA AGGTCGTCAT ATGGCTAGCA	180
	TGGCTAGATT ATTATCTACA TTTACTGAAT ATATTAAGAA TATTATTAAT ACTTCTATAT	240
5	TGAATTTAAG ATATGAAAGT AATCATTTAA TAGACTTATC TAGGTATGCA TCAAAAATAA	300
	ATATTGGTAG TAAAGTAAAT TTTGATCCAA TAGATAAAAA TCAAATTCAA TTATTTAATT	360
10	TAGAAAGTAG TAAAATTGAG GTAATTTTAA AAAATGCTAT TGTATATAAT AGTATGTATG	420
	AAAATTTTAG TACTAGCTTT TGGATAAGAA TTCCTAAGTA TTTTAACAGT ATAAGTCTAA	480
	ATAATGAATA TACAATAATA AATTGTATGG AAAATAATTC AGGATGGAAA GTATCACTTA	540
15	ATTATGGTGA AATAATCTGG ACTTTACAGG ATACTCAGGA AATAAAACAA AGAGTAGTTT	600
	TTAAATACAG TCAAATGATT AATATATCAG ATTATATAAA CAGATGGATT TTTGTAACTA	660
20	TCACTAATAA TAGATTAAAT AACTCTAAAA TTTATATAAA TGGAAGATTA ATAGATCAAA	720
	AACCAATTTC AAATTTAGGT AATATTCATG CTAGTAATAA TATAATGTTT AAATTAGATG	780
	GTTGTAGAGA TACACATAGA TATATTTGGA TAAAATATTT TAATCTTTTT GATAAGGAAT	840
25	TAAATGAAAA AGAAATCAAA GATTTATATG ATAATCAATC AAATTCAGGT ATTTTAAAAG	900
	ACTTTTGGGG TGATTATTTA CAATATGATA AACCATACTA TATGTTAAAT TTATATGATC	960
30	CAAATAAATA TGTCGATGTA AATAATGTAG GTATTAGAGG TTATATGTAT CTTAAAGGGC	1020
	CTAGAGGTAG CGTAATGACT ACAAACATTT ATTTAAATTC AAGTTTGTAT AGGGGGACAA	1080
	AATTTATTAT AAAAAAATAT GCTTCTGGAA ATAAAGATAA TATTGTTAGA AATAATGATC	1140
35	GTGTATATAT TAATGTAGTA GTTAAAAATA AAGAATATAG GTTAGCTACT AATGCATCAC	1200
	AGGCAGGCGT AGAAAAAATA CTAAGTGCAT TAGAAATACC TGATGTAGGA AATCTAAGTC	1260
40	AAGTAGTAGT AATGAAGTCA AAAAATGATC AAGGAATAAC AAATAAATGC AAAATGAATT	1320
	TACAAGATAA TAATGGGAAT GATATAGGCT TTATAGGATT TCATCAGTTT AATAATATAG	1380
	CTAAACTAGT AGCAAGTAAT TGGTATAATA GACAAATAGA AAGATCTAGT AGGACTTTGG	1440
45	GTTGCTCATG GGAATTTATT CCTGTAGATG ATGGATGGGG AGAAAGGCCA CTGTAATTAA	1500
	TCTCAAACTA CATGAGTCTG TCAAGAAGCT TGCGGCCGCA CTCGAG	1546
50	(2) INFORMATION FOR SEQ ID NO:32:	
	(i) SEQUENCE CHARACTERISTICS:(A) LENGTH: 9 amino acids	
<i>-</i> -	(B) TYPE: amino acid (C) STRANDEDNESS: not relevant	
55	(D) TOPOLOGY: not relevant	
	(ii) MOLECULE TYPE: peptide	
60	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:	
	Met His His His His His Met Ala 1 5	
, -	(2) INFORMATION FOR SEQ ID NO:33:	
65	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 21 base pairs (B) TYPE: nucleic acid	
70	(C) STRANDEDNESS: single (D) TOPOLOGY: linear	

	(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA"														
5	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:														
J	TATGCATCAC CATCACCATC A	21													
	(2) INFORMATION FOR SEQ ID NO:34:														
10	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 23 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear														
15	(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA"														
20	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:														
20	CATGTGATGG TGATGGTGAT GCA	23													
	(2) INFORMATION FOR SEQ ID NO:35:	23													
25	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 1351 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: double (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid														
30	<pre>(A) LENGTH: 1351 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: double (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (ix) FEATURE:</pre>														
	(A) DESCRIPTION: /desc = "DNA"														
35	(ix) FEATURE: (A) NAME/KEY: CDS (B) LOCATION: 11335														
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:														
40	ATG CAT CAC CAT CAC CAT CAC ATG GCT CGT CTG CTG TCT ACC TTC ACT Met His His His His His Met Ala Arg Leu Leu Ser Thr Phe Thr 1 5 10	48													
45	GAA TAC ATC AAG AAC ATC ATC AAT ACC TCC ATC CTG AAC CTG CGC TAC Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn Leu Arg Tyr 20 25 30	96													
50	GAA TCC AAT CAC CTG ATC GAC CTG TCT CGC TAC GCT TCC AAA ATC AAC Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser Lys Ile Asn 35 40	144													
55	ATC GGT TCT AAA GTT AAC TTC GAT CCG ATC GAC AAG AAT CAG ATC CAG Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn Gln Ile Gln 50 55 60	192													
	CTG TTC AAT CTG GAA TCT TCC AAA ATC GAA GTT ATC CTG AAG AAT GCT Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu Lys Asn Ala 65 70 75 80	240													
60	ATC GTA TAC AAC TCT ATG TAC GAA AAC TTC TCC ACC TCC TTC TGG ATC Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp Ile 85 90 95	288													
65	CGT ATC CCG AAA TAC TTC AAC TCC ATC TCT CTG AAC AAT GAA TAC ACC Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn Glu Tyr Thr 100 105 110	336													
70	ATC ATC AAC TGC ATG GAA AAC AAT TCT GGT TGG AAA GTA TCT CTG AAC Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val Ser Leu Asn 115	384													

•	TAC Tyr	GGT Gly 130	014	ATO	ATC	TGG Trp	ACT Thr 135	CTG Leu	CAG Gln	GAC Asp	ACT Thr	CAC Glr 140	ı Glu	ATC	Lys	CAG Gln		432
, 5 ,	CGT Arg 145		GTA Val	TTC Phe	AAA Lys	TAC Tyr 150	TCT Ser	CAG Gln	ATG Met	ATC	AAC Asn 155	ATC	TCI Ser	GAC Asp	TAC Tyr	ATC Ile 160		480
10	AAT Asn	CGC Arg	TGG Trp	ATC	TTC Phe 165	GTT Val	ACC Thr	ATC Ile	ACC Thr	AAC Asn 170	Asn	CGT Arg	CTG	AAT Asn	AAC Asn 175	TCC		528
15	AAA Lys	ATC Ile	TAC Tyr	ATC Ile 180	AAC Asn	GGC Gly	CGT Arg	CTG Leu	ATC Ile 185	GAC Asp	CAG Gln	AAA Lys	CCG Pro	ATC Ile 190	TCC Ser	AAT Asn		576
20		7	195			AIG	261	200	ASII	11e	Met	Phe	Lys 205	Leu	Asp	GGT Gly	,	624
2.5	-75	210	Lap	1111	CAC His	Arg	215	11e	Trp	IIe	Lys	Tyr 220	Phe	Asn	Leu	Phe	•	672
25	225	2,3	Giu	Dea	AAC Asn	230	rys	GIU	He	ĻŅS	Asp 235	Leu	Tyr	Asp	Asn	Gln 240	•	720
30			501	Gly	ATC Ile 245	Leu	LYS	Asp	Pne	250	Gly	Asp	Tyr	Leu	Gln 2 5 5	Tyr	-	768
35	,,,,	2,5	110	260	TAC Tyr	met	Leu	ASN	265	Tyr	Asp	Pro	Asn	Lys 270	Tyr	Val	ε	316
40			275	ASII	GTA Val	GIY	116	280	GIY	Tyr	Met	Tyr	Leu 285	Lys	Gly	Pro	ε	864
		290	Jei	vaı	ATG Met	Int	295	Asn	116	Tyr	Leu	300	Ser	Ser	Leu	Tyr	9	912
45	305	GIŸ	1111	Lys	TTC Phe	310	iie	ьуs	Lys	Tyr	Ala 315	Ser	Gly	Asn	Lys	Asp 320	9	60
50	AAT Asn	ATC 11e	GTT Val	CGC Arg	AAC Asn 325	ASI	Asp	Arg	GTA Val	Tyr	Ile	AAT Asn	GTT Val	GTA Val	GTT Val 335	AAG Lys	10	800
55	11511	D y5	GIU	340	CGT Arg	Leu	AIA	Thr	345	Ala	Ser	Gln	Ala	Gly 350	Val	Glu	10	56
60	AAG Lys	ATC Ile	TTG Leu 355	TCT Ser	GCT Ala	CTG Leu	GAA Glu	ATC Ile 360	CCG Pro	GAC Asp	GTT Val	GGT Gly	AAT Asn 365	CTG Leu	TCT Ser	CAG Gln	11	04
	GTA Val	GTT Val 370	GTA Val	ATG Met	AAA Lys	ser	AAG Lys 375	AAC Asn	GAC Asp	CAG Gln	GGT Gly	ATC Ile 380	ACT Thr	AAC Asn	AAA Lys	TGC Cys	11	52
65	AAA Lys 385	ATG Met	AAT Asn	CTG Leu	GIU	GAC Asp 390	AAC Asn	AAT Asn	GGT Gly	AAC Asn	GAT Asp 395	ATC Ile	GGT Gly	TTC Phe	ATC Ile	GGT Gly 400	12	00
70	TTC Phe	CAC His	CAG Gln	TTC Phe	AAC Asn	AAT Asn	ATC Ile	GCT Ala	AAA Lys	CTG Leu	GTT Val	GCT Ala	TCC Ser	AAC Asn	TGG Trp	TAC Tyr	12	48

	405 410 415	
5	AAT CGT CAG ATC GAA CGT TCC TCT CGC ACT CTG GGT TGC TCT TGG GAG Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys Ser Trp Glu 420 425 430	1296
10	TTC ATC CCG GTT GAT GAC GGT TGG GGT GAA CGT CCG CTG TAACCCGGGA Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu 435 440 445	1345
	AAGCTT	1351
	(2) INFORMATION FOR SEQ ID NO:36:	
15	 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 445 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear 	
20	(ii) MOLECULE TYPE: protein	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:	
25	Met His His His His His Met Ala Arg Leu Leu Ser Thr Phe Thr 1 5 10 15	
	Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn Leu Arg Tyr 20 25 30	
30	Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser Lys Ile Asn 35 40 45	
35	Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn Gln Ile Gln 50 60	
	Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu Lys Asn Ala 65 70 75 80	
40	Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp Ile 85 90 95	
	Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn Glu Tyr Thr	
45	Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val Ser Leu Asn 115 120 125	
50	Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu Ile Lys Gln 130 135 140	
50	Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser Asp Tyr Ile 145 150 155 160	
55	Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Asn Asn Ser 165 170 175	
	Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser Asn 180 185 190	
60	Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys Leu Asp Gly 195 200 205	
65	Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe Asn Leu Phe 210 215 220	
	Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Asn Gln 225 235 240	
70	Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr Leu Gln Tyr 245 250 255	

- 281 -

	Asp	Lys	Pro	Tyr 260	Tyr	Met	Leu	Asn	Leu 265	Tyr	Asp	Pro	Asn	Lys 270	Tyr	Val	
5	Asp	Val	Asn 275	Asn	Val	Gly	Ile	Arg 280	Gly	Tyr	Met	Tyr	Leu 285	Lys	Gly	Pro	
	Arg	Gly 290	Ser	Val	Met	Thr	Thr 295	Asn	Ile	Tyr	Leu	Asn 300	Ser	Ser	Leu	Tyr	
10	Arg 305	Gly	Thr	Lys	Phe	Ile 310	Ile	Lys	Lys	Tyr	Ala 315	Ser	Gly	Asn	Lys	Asp 320	
15	Asn	Ile	Val	Arg	Asn 325	Asn	Asp	Arg	Val	Tyr 330	Ile	Asn	Val	Val	Val 335	Lys	
	Asn	Lys	Glu	Tyr 340	Arg	Leu	Ala	Thr	Asn 345	Ala	Ser	Gln	Ala	Gly 350	Val	Glu	
20	Lys	Ile	Leu 355	Ser	Ala	Leu	Glu	Ile 360	Pro	Asp	Val	Gly	Asn 365	Leu	Ser	Gln	
	Val	Val 370	Val	Met	Lys	Ser	Lys 375	Asn	Asp	Gln	Gly	Ile 380	Thr	Asn	Lys	Cys	
25	1.ys 385	Met	Asn	Leu	Gln	Asp 390	Asn	Asn	Gly	Asn	Asp 395	Ile	Gly	Phe	Ile	Gly 400	
30	Phe	liis	Gln	Phe	Asn 405	Asn	Ile	Ala	Lys	Leu 410	Val	Ala	Ser	Asn	Trp 415	Tyr	
	Asn	Arg	Gln	Ile 420	Glu	Arg	Ser	Ser	Arg 425	Thr	Leu	Gly	Cys	Ser 430	Trp	Glu	
35	Phe	11e	Pro 435	Val	Asp	Asp	Gly	Trp 440	Gly	Glu	Arg	Pro	Leu 445				
	(2)	'INFO	RMAT	NOI	FOR	SEQ	ID N	10:37	· ':								
40		(i)	(E	OUENCE A) LE B) TY C) SI O) TO	NGTH PE: RAND	I: 27 nucl EDNE	bas eic SS:	se pa acid	irs								
15		(ii)	MOL (A	ECUL	E TY	PE:	othe N:/	er nu desc	clei	c ac	id						
		(xi)	SEQ	UENC	E DE	SCRI	PTIC	on: s	EQ I	D NO	:37:						
50	CGC	TATA	AA T	ATTC	GTCC	TT A	GCAT	CG									27
	(2)	INFO	RMAT	NOI	FOR	SEQ	ID N	10:38	:								
55		(i)	(B	OUENC L) LE L) TY L) ST L) TC	NGTH PE: RAND	: 27 nucl EDNE	bas eic SS:	e pa acid sino	irs								
50		(ii)	MOL (A	ECUL	E TY	PE: PTIO	othe N:/	r nu desc	clei = "	c ac DNA"	id						
		(xi)	SEQ	UENC	E DE	SCRI	PTIO	N: S	EQ I	D NO	:38:						
55	GGA.	AGCTT	GC A	GGGC	AATT	A CA	TCAT	'G									27
	(2)	INFO	RMAT	NOI	FOR	SEQ	ID N	10 : 3 9	:								
70		(i)	SEQ (A	UENC	E CH	ARAC	TERI 76 b	STIC	S: pair	s							

	(B) TYPE: nucleic acid (C) STRANDEDNESS: double (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic)																
5		(ii) MO	LECU	LE T	YPE:	DNA	(ge	nomi	c)							
		(ix	(AME/	KEY: ION:		3873						t			
10		(xi) SE	QUEN	CE D	ESCR	IPTI:	ON:	SEQ	ID N	0:39	:					
15	ATG Met 1	Pro	GTT Val	ACA Thr	ATA Ile 5	AAT Asn	AAT Asn	TTT Phe	AAT Asn	TAT Tyr 10	AAT Asn	GAT Asp	CCT Pro	ATT Ile	GAT Asp 15	AAT Asn	48
20	GAC Asp	AAT Asn	ATT Ile	ATT Ile 20	ATG Met	ATG Met	GAA Glu	CCT Pro	CCA Pro 25	TTT Phe	GCA Ala	AGG Arg	GGT Gly	ACG Thr 30	GGG Gly	AGA Arg	96
	TAT Tyr	TAT Tyr	AAA Lys 35	GCT Ala	TTT Phe	AAA Lys	ATC Ile	ACA Thr 40	GAT Asp	CGT Arg	ATT Ile	TGG Trp	ATA Ile 45	ATA Ile	CCC Pro	GAA Glu	144
25	AGA Arg	TAT Tyr 50	ACT Thr	TTT Phe	GGA Gly	TAT Tyr	AAA Lys 55	CCT Pro	GAG Glu	GAT Asp	TTT Phe	AAT Asn 60	AAA Lys	AGT Ser	TCC Ser	GGT Gly	192
30	ATT Ile 65	TTT Phe	AAT Asn	AGA Arg	GAT Asp	GTT Val 70	TGT Cys	GAA Glu	TAT Tyr	TAT Tyr	GAT Asp 75	CCA Pro	GAT Asp	TAC Tyr	TTA Leu	AAT Asn 80	240
35	ACC Thr	AAT Asn	GAT Asp	AAA Lys	AAG Lys 85	AAT Asn	ATA Ile	TTT Phe	TTC Phe	CAA Gln 90	ACA Thr	TTG Leu	ATC Ile	AAG Lys	TTA Leu 95	TTT Phe	288
40	AAT Asn	AGA Arg	ATC Ile	AAA Lys 100	TCA Ser	AAA Lys	CCA Pro	TTG Leu	GGT Gly 105	GAA Glu	AAG Lys	TTA Leu	TTA Leu	GAG Glu 110	ATG Met	ATT Ile	336
	ATA Ile	AAT Asn	GGT Gly 115	ATA Ile	CCT Pro	TAT Tyr	CTT Leu	GGA Gly 120	GAT Asp	AGA Arg	CGT Arg	GTT Val	CCA Pro 125	CTC Leu	GAA Glu	GAG Glu	384
45	TTT Phe	AAC Asn 130	ACA Thr	AAC Asn	ATT Ile	GCT Ala	AGT Ser 135	GTA Val	ACT Thr	GTT Val	AAT Asn	AAA Lys 140	TTA Leu	ATT Ile	AGT Ser	AAT Asn	432
50	CCA Pro 145	GGA Gly	GAA Glu	Val	Glu	CGA Arg 150	Lys	AAA Lys	GGT Gly	Ile	TTC Phe 155	GCA Ala	AAT Asn	TTA Leu	ATA Ile	ATA Ile 160	480
55	TTT Phe	GGA Gly	CCT Pro	GGG Gly	CCA Pro 165	GTT Val	TTA Leu	AAT Asn	GAA Glu	AAT Asn 170	GAG Glu	ACT Thr	ATA Ile	GAT Asp	ATA Ile 175	GGT Gly	528
60	ATA Ile	CAA Gln	AAT Asn	CAT His 180	TTT Phe	GCA Ala	TCA Ser	AGG Arg	GAA Glu 185	GGC Gly	TTT Phe	GGG Gly	GGT Gly	ATA Ile 190	ATG Met	CAA Gln	576
	ATG Met	AAA Lys	TTT Phe 195	TGT Cys	CCA Pro	GAA Glu	TAT Tyr	GTA Val 200	AGC Ser	GTA Val	TTT Phe	AAT Asn	AAT Asn 205	GTT Val	CAA Gln	GAA Glu	624
65	AAC Asn	AAA Lys 210	GGC Gly	GCA Ala	AGT Ser	ATA Ile	TTT Phe 215	AAT Asn	AGA Arg	CGT Arg	GGA Gly	TAT Tyr 220	TTT Phe	TCA Ser	GAT Asp	CCA Pro	672
70	GCC Ala	TTG Leu	ATA Ile	TTA Leu	ATG Met	CAT His	GAA Glu	CTT Leu	ATA Ile	CAT His	GTT Val	TTG Leu	CAT His	GGA Gly	TTA Leu	TAT Tyr	720

	225	;				230)				235	5				240	
5	•		,-		245	, nap	, Dec	FIC	, 116	250	Pro) Asn	Glu	Lys	Lys 255	TTT Phe	768
10	TTT Phe	Met	Gln	TCT Ser 260	1111	GAT Asp	ACT Thr	ATA Ile	CAG Gln 265	Ala	GAA Glu	GAA Glu	CTA Leu	TAT Tyr 270	Thr	TTT	816
	GGA Gly	GGA Gly	CAA Gln 275	. wah	CCC	AGC Ser	ATC Ile	ATA Ile 280	ser	CCT Pro	TCT Ser	ACA Thr	GAT Asp 285	AAA Lys	AGT Ser	ATC Ile	864
15	TAT Tyr	GAT Asp 290	- Lys	GTT Val	TTG Leu	CAA Gln	AAT Asn 295	Pne	AGG Arg	GGG Gly	ATA Ile	GTT Val 300	GAT Asp	AGA Arg	CTT Leu	AAC Asn	912
20	AAG Lys 305	GTT Val	TTA Leu	GTT Val	TGC Cys	ATA Ile 310	TCA Ser	GAT Asp	CCT Pro	AAC Asn	ATT Ile 315	AAC Asn	ATT Ile	AAT Asn	ATA Ile	TAT Tyr 320	960
25	AAA Lys	AAT Asn	AAA Lys	TTT Phe	AAA Lys 325	GAT Asp	AAA Lys	TAT Tyr	AAA Lys	TTC Phe 330	GTT Val	GAA Glu	GAT Asp	TCT Ser	GAA Glu 335	GGA Gly	1008
30	AAA Lys	TAT Tyr	AGT Ser	ATA Ile 340	GAT Asp	GTA Val	GAA Glu	AGT Ser	TTC Phe 345	AAT Asn	AAA Lys	TTA Leu	TAT Tyr	AAA Lys 350	AGC Ser	TTA Leu	1056
•••	ATG Met	TTA Leu	GGT Gly 355	TTT Phe	ACA Thr	GAA Glu	ATT Ile	AAT Asn 360	ATA Ile	GCA Ala	GAA Glu	AAT Asn	TAT Tyr 365	AAA Lys	ATA Ile	AAA Lys	1104
35	ACT Thr	AGA Arg '370	GCT Ala	TCT	TAT Tyr	TTT Phe	AGT Ser 375	GAT Asp	TCC Ser	TTA Leu	CCA Pro	CCA Pro 380	GTA Val	AAA Lys	ATA Ile	AAA Lys	1152
40	AAT Asn 385	TTA Leu	TTA Leu	GAT Asp	AAT Asn	GAA Glu 390	ATC Ile	TAT Tyr	ACT Thr	ATA Ile	GAG Glu 395	GAA Glu	GGG Gly	TTT Phe	AAT Asn	ATA Ile 400	1200
45	TCT Ser	GAT Asp	AAA Lys	AAT Asn	ATG Met 405	GGA Gly	AAA Lys	GAA Glu	TAT Tyr	AGG Arg 410	GGT Gly	CAG Gln	TAA neA	AAA Lys	GCT Ala 415	ATA Ile	1248
50	AAT Asn	AAA Lys	CAA Gln	GCT Ala 420	TAT Tyr	GAA Glu	GAA Glu	ATC Ile	AGC Ser 425	AAG Lys	GAG Glu	CAT His	TTG Leu	GCT Ala 430	GTA Val	TAT Tyr	1296
	AAG Lys	ATA Ile	CAA Gln 435	ATG Met	TGT Cys	AAA Lys	AGT Ser	GTT Val 440	AAA Lys	GTT Val	CCA Pro	GGA Gly	ATA Ile 445	TGT Cys	ATT Ile	GAT Asp	1344
55	GTC Val	GAT Asp 450	AAT Asn	GAA Glu	AAT Asn	TTG Leu	TTC Phe 455	TTT Phe	ATA Ile	GCT Ala	GAT Asp	AAA Lys 460	AAT Asn	AGT Ser	TTT Phe	TCA Ser	1392
60	GAT Asp 465	GAT Asp	TTA Leu	TCT Ser	AAA Lys	AAT Asn 470	GAA Glu	AGA Arg	GTA Val	GAA Glu	TAT Tyr 475	AAT Asn	ACA Thr	CAG Gln	AAT Asn	AAT Asn 480	1440
65	TAT Tyr	ATA Ile	GGA Gly	AAT Asn	GAC Asp 485	TTT Phe	CCT Pro	ATA Ile	AAT Asn	GAA Glu 490	TTA Leu	ATT Ile	TTA Leu	GAT Asp	ACT Thr 495	GAT Asp	1488
70	TTA Leu	ATA Ile	AGT Ser	AAA Lys 500	ATA Ile	GAA Glu	TTA Leu	CCA Pro	AGT Ser 505	GAA Glu	AAT Asn	ACA Thr	Glu	TCA Ser 510	CTT Leu	ACT Thr	1536

	GAT Asp	TTT Phe	AAT Asn 515	vaı	GAT Asp	GTT Val	CCA Pro	GTA Val	Tyr	GAA Glu	AAA Lys	CAA Glm	CCC Pro	Ala	ATA	AAA Lys	1584
5	AAA Lys	GTT Val 530	Pne	ACA Thr	GAT Asp	GAA Glu	AAT Asn 535	Thr	ATC	TTT Phe	CAA Gln	TAT Tyr 540	Leu	TAC	TCT Ser	CAG Gln	1632
10	ACA Thr 545	ипе	CCT Pro	CTA Leu	AAT Asn	ATA Ile 550	AGA Arg	GAT Asp	ATA Ile	AGT Ser	TTA Leu 555	Thr	TCT Ser	TCA Ser	TTT Phe	GAT Asp 560	1680
15	Asp		Leu	Leu	565	ser	Ser	Lys	Val	Tyr 570	Ser	Phe	Phe	Ser	Met 575	Asp	1728
20	Tyt	116	Lys	580	Ala	Asn	Lys	Val	Va1 585	Glu	Ala	Gly	Leu	Phe 590	Ala	GGT Gly	1776
•	irp	GTG Val	595	GIN	116	vaı	Asp	Asp 600	Phe	Val	Ile	Glu	Ala 605	Asn	Lys	Ser	1824
25	ser	ACT Thr 610	мес	Asp	Lys	116	615	Asp	Ile	Ser	Leu	Ile 620	Val	Pro	Tyr	Ile	1872
30	625	TTA Leu	Ala	Leu	Asn	Val 630	Gly	Asp	Glu	Thr	Ala 635	Lys	Gly	Asn	Phe	Glu 640	1920
35	ser	GCT Ala	Pne	GIu	11e 645	Ala	GΙΆ	Ser	Ser	11e 650	Leu	Leu	Glu	Phe	11e 655	Pro	1968
40	GIU	CTT Leu	Leu	660	Pro	Val	Val	Gly	Val 665	Phe	Leu	Leu	Glu	Ser 670	Tyr	Ile	2016
	Asp	AAT Asn	Lys 675	Asn	Lys	He	Ile	Lys 680	Thr	Ile	Asp	Asn	Ala 685	Leu	Thr	Lys	2064
45	Arg	GTG Val 690	Glu	Lys	Trp	Ile	Asp 695	Met	Tyr	Gly	Leu	11e 700	Val	Ala	Gln	Trp	2112
50	705	TCA Ser	Thr	Val	Asn	710	Gln	Phe	Tyr	Thr	11e 715	Lys	Glu	Gly	Met	Tyr 720	2160
55	Lys	GCT Ala	Leu	Asn	725	Gin	Ala	Gln	Ala	Leu 730	Glu	Glu	Ile	Ile	Lys 735	Tyr	2208
60	ьys	TAT Tyr	Asn	740	Tyr	Ser	Glu	Glu	Glu 745	Lys	Ser	Asn	Ile	Asn 750	Ile	Asn	2256
	TTT Phe	AAT Asn	GAT Asp 755	ATA Ile	TAA NEA	TCT Ser	AAA Lys	CTT Leu 760	AAT Asn	GAT Asp	GGT Gly	ATT Ile	AAC Asn 765	CAA Gln	GCT Ala	ATG Met	2304
65	GAT Asp	AAT Asn 770	ATA Ile	AAT Asn	GAT Asp	Phe	ATA Ile 775	AAT Asn	GAA Glu	TGT Cys	TCT Ser	GTA Val 780	TCA Ser	TAT Tyr	TTA Leu	ATG Met	2352
70	AAA Lys	AAA Lys	ATG Met	ATT Ile	CCA Pro	TTA Leu	GCT Ala	GTA Val	AAA Lys	AAA Lys	TTA Leu	CTA Leu	GAC Asp	TTT Phe	GAT Asp	AAT Asn	2400

	785					790					795					800	
5	ACT Thr	CTC Leu	AAA Lys	AAA Lys	AAT Asn 805	nen	TTA Leu	AAT Asn	TAT Tyr	ATA 1le 810	GAT Asp	GAA Glu	AAT Asn	AAA Lys	TTA Leu 815	TAT	2448
10	TTA Leu	ATT Ile	GGA Gly	AGT Ser 820	GTA Val	GAA Glu	GAT Asp	GAA Glu	AAA Lys 825	TCA Ser	AAA Lys	GTA Val	GAT Asp	AAA Lys 830		TTG Leu	2496
•	AAA Lys	ACC Thr	ATT Ile 835	ATA Ile	CCA Pro	TTT Phe	GAT Asp	CTT Leu 840	Ser	ACG Thr	TAT Tyr	TCT Ser	AAT Asn 845	ATT Ile	GAA Glu	ATA Ile	2544
15	CTA Leu	ATA Ile 850	AAA Lys	ATA Ile	TTT Phe	AAT Asn	AAA Lys 855	TAT Tyr	AAT Asn	AGC Ser	GAA Glu	ATT Ile 860	TTA Leu	AAT Asn	AAT Asn	ATT Ile	2592
20	ATC Ile 865	TTA Leu	AAT Asn	TTA Leu	AGA Arg	TAT Tyr 870	AGA Arg	GAT Asp	AAT Asn	AAT Asn	TTA Leu 875	ATA Ile	GAT Asp	TTA Leu	TCA Ser	GGA Gly 880	2640
25	TAT Tyr	GGA Gly	GCA Ala	AAG Lys	GTA Val 885	GAG Glu	GTA Val	TAT Tyr	GAT Asp	GGG Gly 890	GTC Val	AAG Lys	CTT Leu	AAT Asn	GAT Asp 895	AAA Lys	2688
30	AAT Asn	CAA Gln	TTT Phe	AAA Lys 900	TTA Leu	ACT Thr	AGT Ser	TCA Ser	GCA Ala 905	GAT Asp	AGT Ser	AAG Lys	ATT	AGA Arg 910	GTC Val	ACT Thr	2736
	CAA Gln	AAT Asn	CAG Gln 915	AAT Asn	ATT Ile	ATA Ile	TTT Phe	AAT Asn 920	AGT Ser	ATG Met	TTC Phe	CTT Leu	GAT Asp 925	TTT Phe	AGC Ser	GTT Val	2784
35		930	.rp	ATA Ile	Arg	116	935	Lys	Tyr	Arg	Asn	Asp 940	Asp	Ile	Gln	Asn	2832
40	945	110	5	AAT Asn	Giu	950	Thr	11e	lle	Asn	Cys 955	Met	Lys	Asn	Asn	Ser 960	2880
45	GGC Gly	111	Lys	116	965	iie	Arg	GIŸ	Asn	970	Ile	lie	Trp	Thr	Leu 975	Ile	2928
50	GAT Asp	116	ASII	980	ràs	inr	Lys	Ser	985	Phe	Phe	Glu	Tyr	Asn 990	Ile	Arg	2976
	GAA Glu	тор	995	361	GIU	Tyr	iie	1000	Arg	Trp	Phe	Phe	Val 1005	Thr	Ile	Thr	3024
55	AAT Asn	1010	Leu	Asp	ASN	АТА	Lys 1015	Ile	Tyr	Ile	Asn	Gly 1020	Thr	Leu	Glu	Ser	3072
60	AAT Asn 1025	rice	vsb	116	Lys	ASP 1030	11e	GIÀ	GLu	Val	Ile 1035	Val	naA	Gly	Glu	Ile 1 04 0	3120
65	ACA Thr	rne	Lys	reu	ASP 1045	GIY	Asp	Val	Asp	Arg 1050	Thr	Gln	Phe	Ile	Trp 1055	Met	3168
70	AAA Lys	TAT Tyr	FILE	AGT Ser 1060	116	TTT Phe	AAT Asn	ACG Thr	CAA Gln 1065	Leu	AAT Asn	CAA Gln	Ser	AAT Asn 1070	Ile	AAA Lys	3216

	GAG ATA TAT AAA ATT CAA TCA TAT AGC GAA TAC TTA AAA GAT TTT TGG Glu Ile Tyr Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp	3264
. 5	GGA AAT CCT TTA ATG TAT AAT AAA GAA TAT TAT ATG TTT AAT GCG GGG Gly Asn Pro Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly	3312
10	AAT AAA AAT TCA TAT ATT AAA CTA GTG AAA GAT TCA TCT GTA GGT GAA Asn Lys Asn Ser Tyr Ile Lys Leu Val Lys Asp Ser Ser Val Gly Glu	3360
15	ATA TTA ATA CGT AGC AAA TAT AAT CAG AAT TCC AAT TAT ATA AAT TAT Ile Leu Ile Arg Ser Lys Tyr Asn Gln Asn Ser Asn Tyr Ile Asn Tyr 1125 1130 1135	3408
20	AGA AAT TTA TAT ATT GGA GAA AAA TTT ATT A	3456
5-	TCT CAA TCT ATA AAT GAT GAT ATA GTT AGA AAA GAA GA	3504
25	CTA GAT TTG GTA CTT CAC CAT GAA GAG TGG AGA GTA TAT GCC TAT AAA Leu Asp Leu Val Leu His His Glu Glu Trp Arg Val Tyr Ala Tyr Lys 1170 1180	3552
30	TAT TTT AAG GAA CAG GAA GAA AAA TTG TTT TTA TCT ATT ATA AGT GAT Tyr Phe Lys Glu Gln Glu Glu Lys Leu Phe Leu Ser Ile Ile Ser Asp 1185 1190 1195 1200	3600
35	TCT AAT GAA TTT TAT AAG ACT ATA GAA ATA AAA GAA TAT GAT GAA CAG Ser Asn Glu Phe Tyr Lys Thr Ile Glu Ile Lys Glu Tyr Asp Glu Gln 1205 1210 1215	3648
40	CCA TCA TAT AGT TGT CAG TTG CTT TTT AAA AAA GAT GAA GAA AGT ACT Pro Ser Tyr Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr 1220 1230	3696
45	GAT GAT ATA GGA TTG ATT GGT ATT CAT CGT TTC TAC GAA TCT GGA GTT ASP ASP Ile Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Val 1235 1240 1245	3744
,	TTA CGT AAA AAG TAT AAA GAT TAT TTT TGT ATA AGT AAA TGG TAC TTA Leu Arg Lys Lys Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu 1250 1260	3792
50	AAA GAG GTA AAA AGG AAA CCA TAT AAG TCA AAT TTG GGA TGT AAT TGG Lys Glu Val Lys Arg Lys Pro Tyr Lys Ser Asn Leu Gly Cys Asn Trp 1265 1270 1275 1280	3840
55	CAG TTT ATT CCT AAA GAT GAA GGG TGG ACT GAA TAA Gln Phe Ile Pro Lys Asp Glu Gly Trp Thr Glu 1285 1290 (2) INFORMATION FOR SEQ ID NO:40:	3876
60	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 1291 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear	
65	(ii) MOLECULE TYPE: protein	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:	
70	Met Pro Val Thr Ile Asn Asn Phe Asn Tyr Asn Asp Pro Ile Asp Asn 1 5 10 15	

	Asp	Asn	Ile	Ile 20	Met	Met	Glu	Pro	Pro 25	Phe	Ala	Arg	Gly	Thr 30	Gly	Arg
5	Tyr	Tyr	Lys 35	Ala	Phe	Lys	Ile	Thr 40	Asp	Arg	Ile	Trp	Ile 45	Ile	Pro	Glu
	Arg	Tyr 50	Thr	Phe	Gly	туr	Lys 55	Pro	Glu	Asp	Phe	Asn 60	Lys	Ser	Ser	Gly
10	11e 65	Phe	Asn	Arg	Asp	Val 70	Cys	Glu	Tyr	Tyr	Asp 75	Pro	Asp	Tyr	Leu	Asr 80
15	Thr	Asn	Asp	Lys	Lys 85	Asn	Ile	Phe	Phe	Gln 90	Thr	Leu	Ile	Lys	Leu 95	Ph∈
	Asn	Arg	Ile	Lys 100	Ser	Lys	Pro	Leu	Gly 105	Glu	Lys	Leu	Leu	Glu 110	Met	ile
20	lle	Asn	Gly 115	Ile	Pro	Tyr	Leu	Gly 120	Asp	Arg	Arg	Val	Pro 125	Leu	Glu	Glu
	Phe	Asn 130	Thr	Asn	Ile	Ala	Ser 135	Val	Thr	Val	Asn	Lys 140	Leu	Ile	Ser	Asn
25	Pro 145	Gly	Glu	Val	Glu	Arg 150	Lys	Lys	Gly	Ile	Phe 155	Ala	Asn	Leu	Ile	11e
30	Phe	Gly	Pro	Gly	Pro 165	Val	Leu	Asn	Glu	Asn 170	Glu	Thr	Ile	Asp	Ile 175	Gly
	lle	Gln	Asn	His 180	Phe	Ala	Ser	Arg	Glu 185	Gly	Phe	Gly	Gly	Ile 190	Met	Gln
35	Met	Lys	Phe 195	Cys	Pro	Glu	Tyr	Val 200	Ser	Val	Phe	Asn	Asn 205	Val	Gln	Glu
	Asn	'Lys 210	Gly	Ala	Ser	Ile	Phe 215	Asn	Arg	Arg	Gly	Tyr 220	Phe	Ser	Asp	Pro
40	Ala 225	Leu	Ile	Leu	Met	His 230	Glu	Leu	Ile	His	Val 235	Leu	His	Gly	Leu	Tyr 240
45	Gly	Ile	Lys	Val	Asp 245	Asp	Leu	Pro	Ile	Val 250	Pro	Asn	Glu	Lys	Lys 255	Phe
	Phe	Met	Gln	Ser 260	Thr	Asp	Thr	Ile	Gln 265	Ala	Glu	Glu	Leu	Tyr 270	Thr	Phe
50	Gly	Gly	Gln 275	Asp	Pro	Ser	Ile	11e 280	Ser	Pro	Ser	Thr	Asp 285	Lys	Ser	Ile
	Tyr	Asp 290	Lys	Val	Leu	Gln	Asn 295	Phe	Arg	Gly	Ile	Val 300	qeA	Arg	Leu	Asn
55	Lys 305	Val	Leu	Val	Суѕ	Ile 310	Ser	Asp	Pro	Asn	Ile 315	Asn	Ile	Asn	Ile	Tyr 320
60	Lys	Asn	Lys	Phe	Lys 325	Asp	Lys	Tyr	Lys	Phe 330	Val	Glu	Asp	Ser	Glu 335	Gly
(V)	Lys	Tyr	Ser	11e 340	Asp	Val	Glu	Ser	Phe 345	Asn	Lys	Leu	Tyr	Lys 350	Ser	Leu
65	Met	Leu	Gly 355	Phe	Thr	Glu	Ile	Asn 360	11e	Ala	Glu	Asn	Тут 365	Lys	Ile	Lys
	Thr	Arg 370	Ala	Ser	Tyr	Phe	Ser 375	Asp	Ser	Leu	Pro	Pro 380	Val	Lys	Ile	Lys
70	Asn	Leu	Leu	Asp	Asn	Glu	Ile	Tyr	Thr	İle	Glu	Glu	Gly	Phe	Asn	Ile

	385					390)				399	5				400
5	Ser	Asp	Lys	s Asn	Met 405	Gly	/ Lys	Glu	туі	410	g Gly	Glr G	n Asr	ı Lys	419	
				420	,				425					430)	
10			433)				440	ı	Val			445	i		
15		430					455	'		Ala		460	1			
15	405					470				Glu	475					480
20					485					Glu 490					495	
				500					505					510		
25			313					520		Glu			525			
	-,2	530	1110	1	дар	GIU	535	1111	116	Phe	GIn	Туг 540	Leu	Tyr	Ser	Gln
30	242					550				Ser	555					560
35					202					Tyr 570					575	
				580					585	Glu				590		
40			232					600		Val			605			
45		010					615			Ser		620				
7.7	02.5					630				Thr	635					640
50					645					Ile 650					655	
				000					665					670		
55	Asp		0/5					680					685			_
	Arg	690	Gru	rys	Trp	iie	Asp 695	Met	Tyr	Gly	Leu	11e 700	Val	Ala	Gln	Trp
60	Leu 705					/10					715					720
65	Lys .				725					730					735	
	Lys			/40					745					750		
70	Phe .	Asn	Asp 755	Ile	Asn	Ser	Lys	Leu 760	Asn	Asp	Gly	Ile	Asn 765	Gln	Ala	Met

	Asp	770	тте	Asn	Asp	Phe	775	Asn	Glu	Cys	Ser	Val 780	Ser	Tyr	Leu	Met
5	Lys 785	Lys	Met	Ile	Pro	Leu 790	Ala	Val	Lys	Lys	Leu 795	Leu	Asp	Phe	Asp	Asn 800
	Thr	Leu	Lys	Lys	Asn 805	Leu	Leu	Asn	Tyr	Ile 810	Asp	Glu	Asn	Lys	Leu 815	
10	Leu	Ile	Gly	Ser 820	Val	Glu	Asp	Glu	Lys 825	Ser	Lys	Val	Asp	Lys 830	туг	Leu
15	Lys	Thr	Ile 835	Ile	Pro	Phe	Asp	Leu 840	Ser	Thr	Tyr	Ser	Asn 845	Ile	Ğlu	Ile
	Leu	Ile 850	Lys	Ile	Phe	Asn	Lys 855	Tyr	Asn	Ser	Glu	Ile 860	Leu	Asn	Asn	Ile
20	Ile 865	Leu	Asn	Leu	Arg	Tyr 870	Arg	Asp	Asn	Asn	Leu 875	Ile	Asp	Leu	Ser	Gly 880
	Tyr	Gly	Ala	Lys	Val 885	Glu	Val	Tyr	Asp	Gly 890	Val	Lys	Leu	Asn	Asp 895	Lys
25	Asn	Gln	Phe	Lys 900	Leu	Thr	Ser	Ser	Ala 905	Asp	Ser	Lys	Ile	Arg 910	Val	Thr
30			713		Ile			920					925			
	Ser	Phe 930	Trp	Ile	Arg	Ile	Pro 935	Lys	Tyr	Arg	Asn	Asp 940	Asp	Ile	Gln	Asn
35	74.3				Glu	950					955					960
	Gly	Trp	Lys	Ile	Ser 965	Ile	Arg	Gly	Asn	Arg 970	Ile	Ile	Trp	Thr	Leu 975	Ile
40	Λsp	Ile	Asn	Gly 980	Lys	Thr	Lys	Ser	Val 985	Phe	Phe	Glu	Tyr	Asn 990	Ile	Arg
45	Glu	Asp	11e 995	Ser	Glu	Tyr	Ile	Asn 1000	Arg	Trp	Phe	Phe	Val 1005		Ile	Thr
	Asn	Asn 1010	Leu	Asp	Asn	Ala	Lys 1015	Ile	Tyr	lle	Asn	Gly 1020	Thr	Leu	Glu	Ser
50	Asn 1025	Met	Asp	Ile	Lys	Asp 1030	Ile)	Gly	Glu	Val	Ile 1039	Val	Λsn	Gly	Glu	Ile 1040
	Thr	Phe	Lys	Leu	Asp 1045	Gly	Asp	Val	qaA	Arg 1050	Thr	Gln	Phe	Ile	Trp 1055	
55	Lys	Tyr	Phe	Ser 1060	Ile	Phe	Asn	Thr	Gln 1065	Leu	Asn	Gln	Ser	Asn 1070		Lys
60			10/5	li .	Ile			1080)				1085			
	Gly	Asn 1090	Pro	Leu	Met	Tyr	Asn 1095	Lys	Glu	Tyr	Tyr	Met 1100	Phe	Asn	Ala	Gly
65	Asn 1105	Lys	Asn	Ser	Tyr	Ile 1110	Lys)	Leu	Val	Lys	Asp 1115	Ser	Ser	Val	Gly	Glu 1120
	Ile	Leu	Ile	Arg	Ser 1125	Lys	Tyr	Asn	Gln	Asn 1130	Ser	Asn	Tyr	ſle	Asn 1135	
70	Arg	Asn	Leu	Tyr	Ile	Gly	Glu	Lys	Phe	Ile	Ile	Arg	Arg	Glu	Ser	Asn

				114	0				114	5				115	0		
5	Ser	Gln	Ser 115	Ile 5	Asn	Asp	Asp	Ile 116	Val 0	Arg	Lys	Glu	Asp 116		Ile	His	
•	Leu	Asp 117	Leu 0	Val	Leu	His	His 117	Glu 5	Glu	Trp	Arg	Val 118		Ala	туr	Lys	
10	Tyr 118	Phe 5	Lys	·Glu	Gln	Glu 119	Glu 0	Lys	Leu	Phe	Leu 119		Ile	Ile	Ser	Asp 1200	
	Ser	Asn	Glu	Phe	Tyr 120	Lys 5	Thr	Ile	Glu	Ile 121		Glu	Tyr	Asp	G l u 121	Gln 5	
15	Pro	Ser	Tyr	Ser 122	Cys 0	Gln	Leu	Leu	Phe 122		Lys	Asp	Glu	Glu 123		Thr	
20	Λsp	Asp	Ile 123	Gly 5	Leu	Ile	Gly	Ile 124	His O	Arg	Phe	Tyr	Glu 124		Gly	Val	
	Leu	Arg 1250	Lys)	Lys	Tyr	Lys	Asp 125		Phe	Cys	Ile	Ser 126		Trp	Tyr	Leu	
25	Lys 126	Glu 5	Val	Lys	Arg	Lys 127	Pro 0	Tyr	Lys	Ser	Asn 127		Gly	Cys	Asn	Trp 1280	
	Gln	Phe	lle	Pro	Lys 1289		Glu	Gly	Trp	Thr 129							
30	(2)	INFO	ORMA'	rion	FOR	SEQ	ID I	NO : 4	1:								
35	•	(i)	() () ()	QUENCA) LI B) TY C) ST O) TO	ENGTI YPE : TRANI	i: 36 nuc: DEDNI	B76 l leic ESS:	oase acid doul	pai: d	rs							
40			FEA	LECUI ATURE A) NA B) LO	E: Ame/f	KEY:	CDS		nomi	c)							
45		(xi)	SE	QUENC	CE DE	ESCRI	IPTIC	ON: 5	SEQ :	ID NO	D:41	:					
	ΛTG Met 1	CCA Pro	GTT Val	ACA Thr	ATA Ile 5	AAT Asn	AAT Asn	TTT Phe	AAT Asn	TAT Tyr 10	AAT Asn	GAT Asp	CCT Pro	ATT Ile	GAT Asp 15	AAT Asn	48
50	AAT Asn	AAT Asn	ATT Ile	ATT Ile 20	ATG Met	ATG Met	GAG Glu	CCT Pro	CCA Pro 25	TTT Phe	GCG Ala	AGA Arg	GGT Gly	ACG Thr 30	GGG Gly	AGA Arg	96
55	TAT Tyr	TAT Tyr	AAA Lys 35	GCT Ala	TTT Phe	AAA Lys	ATC Ile	ACA Thr 40	GAT Asp	CGT Arg	ATT Ile	TGG Trp	ATA Ile 45	ATA Ile	CCG Pro	GAA Glu	144
60	AGA Arg	TAT Tyr 50	ACT Thr	TTT Phe	GGA Gly	TAT Tyr	AAA Lys 55	CCT Pro	GAG Glu	GAT Asp	TTT Phe	AAT Asn 60	AAA Lys	AGT Ser	TCC Ser	GGT Gly	192
65	ATT Ile 65	TTT Phe	AAT Asn	AGA Arg	GAT Asp	GTT Val 70	TGT Cys	GAA Glu	TAT Tyr	TAT Tyr	GAT Asp 75	CCA Pro	GAT Asp	TAC Tyr	TTA Leu	TAA Asn 80	240
	ACT Thr	AAT Asn	GAT Asp	AAA Lys	AAG Lys 85	AAT Asn	ATA Ile	TTT Phe	TTA Leu	CAA Gln 90	ACA Thr	ATG Met	ATC Ile	AAG Lys	TTA Leu 95	TTT Phe	288
70	AAT	AGA	ATC	AAA	TCA	AAA	CCA	TTG	GGT	GAA	AAG	TTA	ATT	GAG	ATG	TTA	336

- 291 -

	Asn	Arg	, Ile	Lys 100	Ser	Lys	Pro	Leu	Gly 105	Glu	Lys	Leu	Leu	Glu 110	Met	: Ile	
5	ATA Ile	IAA . naA :	GGT Gly 115	-1-	CCT Pro	TAT Tyr	CTT Leu	GGA Gly 120	Asp	AGA Arg	CGT Arg	GTT Val	CCA Pro 125	Leu	GAA Glu	GAG Glu	384
10		130		A311	116	AIG	135	vaı	Thr	Vai	Asn	Lys 140	Leu	lle	Ser	AAT Asn	432
15	145	U .,	o _{zu}	vai	GIU	150	Lys	ьуs	GIA	ile	Phe 155	Ala	Asn	Leu	Ile	160	480
-			CCT Pro	dry	165	Val	Leu	AST	GIU	170	Glu	Thr	Ile	Asp	Ile 175	Gly	528
20		011	ASI	180	FIIE	Ala	ser	Arg	185	Gly	Phe	Gly	Gly	11e 190	Met		576
25		2,5	TTT Phe 195	Суб	PIO	GIU	lyr	200	Ser	Val	Phe	Asn	Asn 205	Val	Gln	Glu	624
30		210	GGC Gly	AIA	Ser	116	215	ASN	Arg	Arg	Gly	Tyr 220	Phe	Ser	Asp	Pro	672
35	225	Jeu	ATA Ile	Deu	Mec	230	GIU	ren	116	His	Val 235	Leu	His	Gly	Leu	Tyr 240	720
	GGC Gly	ATT	AAA Lys	GTA Val	GAT Asp 245	GAT Asp	TTA Leu	CCA Pro	ATT Ile	GTA Val 250	CCA Pro	AAT Asn	GAA Glu	ΛΑΑ Lys	AAA Lys 255	TTT Phe	768
40	TTT Phe	ATG Met	CAA Gln	TCT Ser 260	ACA Thr	GAT Asp	GCT Ala	ATA Ile	CAG Gln 265	GCA Ala	GAA Glu	GAA Glu	CTA Leu	TAT Tyr 270	ACA Thr	TTT Phe	816
45	GGA Gly	GGA Gly	CAA Gln 275	GAT Asp	CCC Pro	AGC Ser	λTC Ile	ATA 1le 280	ACT Thr	CCT Pro	TCT Ser	ACG Thr	GAT Asp 285	AAA Lys	AGT Ser	ATC Ile	864
50	TAT Tyr	GAT Asp 290	AAA Lys	GTT Val	TTG Leu	CAA Gln	AAT Asn 295	TTT Phe	AGA Arg	GGG Gly	ATA Ile	GTT Val 300	GAT Asp	AGA Arg	CTT Leu	AAC Asn	912
55	AAG Lys 305	GTT Val	TTA Leu	GTT Val	TGC Cys	ATA Ile 310	TCA Ser	GAT Asp	CCT Pro	AAC Asn	ATT Ile 315	AAT Asn	ATT Ile	AAT Asn	ATA Ile	TAT Tyr 320	960
	AAA Lys	AAT Asn	AAA Lys	TTT Phe	AAA Lys 325	GAT Asp	AAA Lys	TAT Tyr	AAA Lys	TTC Phe 330	GTT Val	GAA Glu	GAT Asp	TCT Ser	GAG Glu 335	GGA Gly	1008
60	AAA l.ys	TAT Tyr	AGT Ser	ATA Ile 340	GAT Asp	GTA Val	GAA Glu	AGT Ser	TTT Phe 345	GAT Asp	AAA Lys	TTA Leu	Tyr	AAA Lys 350	AGC Ser	TTA Leu	1056
65	ATG Met	TTT Phe	GGT Gly 355	TTT Phe	ACA Thr	GAA Glu	inr	AAT Asn 360	ATA Ile	GCA Ala	GAA Glu	AAT Asn	TAT Tyr 365	AAA Lys	ATA Ile	AAA Lys	1104
70	ACT Thr	AGA Arg 370	GCT Ala	TCT Ser	TAT Tyr	Pne	AGT Ser 375	GAT Asp	T CC Ser	TTA Leu	Pro	CCA Pro 380	GTA Val	aaa Lys	ATA Ile	AAA Lys	1152

	AAT Asn 385	Leu	TTA Leu	GAT Asp	AAT Asn	GAA Glu 390	ATC Ile	TAT Tyr	ACT Thr	ATA Ile	GAG Glu 395	GAA Glu	GGG Gly	TTT Phe	AAT Asn	ATA Ile 400	1200
5	TC T Ser	GAT Asp	AAA Lys	GAT Asp	ATG Met 405	GAA Glu	AAA Lys	GAA Glu	TAT Tyr	AGA Arg 410	GGT Gly	CAG Gln	AAT Asn	AAA Lys	GCT Ala 415	ATA Ile	1248
10	AAT Asn	AAA Lys	CAA Gln	GCT Ala 420	TAT Tyr	GAA Glu	GAA Glu	ATT	AGC Ser 425	AAG Lys	GAG Glu	CAT His	TTG Leu	GCT Ala 430	GTA Val	TAT Tyr	1296
15	Lys	Ile	Gln 435	Met	Cys	Lys	Ser	Val 440	Lys	Ala	Pro	Gly	ATA Ile 445	Cys	Ile	Asp	1344
20	vai	450	Asn	GIu	Asp	Leu	Phe 455	Phe	Ile	Ala	Asp	Lys 460	AAT Asn	Ser	Phe	Ser	1392
	465	Asp	Leu	Ser	Lys	470	Glu	Arg	Ile	Glu	Tyr 475	Asn	ACA Thr	Gln	Ser	Asn 480	1440
' 25 '	Tyr	lle	Glu	Asn	485	Phe	Pro	Ile	Asn	Glu 490	Leu	Ile	TTA Leu	Asp	Thr 495	Asp	1488
30	Leu	lle	Ser	Lys 500	Ile	Glu	Leu	Pro	Ser 505	Glu	Asn	Thr	GAA Glu	Ser 510	Leu	Thr	1536
35	Asp	Phe	Asn 515	Val	Asp	Val	Pro	Val 520	Tyr	Glu	Lys	Gln	CCC Pro 525	Ala	Ile	Lys	1584
4()	Lys	530	Phe	Thr	Asp	Glu	Asn 535	Thr	Ile	Phe	Gln	Tyr 540	TTA Leu	Tyr	Ser	Gln	1632
4.5	Thr 545	Phe	Leu	Leu	Asp	11e 550	Arg	Asp	Ile	Ser	Leu 555	Thr	TCT Ser	Ser	Phe	Asp 560	1680
45	Asp	Ala	Leu	Leu	Phe 565	Ser	Asn	Lys	Val	Tyr 570	Ser	Phe	TTT Phe	Ser	Met 575	Asp	1728
50	Tyr	Ile	Lys	Thr 580	Ala	Asn	Lys	Val	Val 585	Glu	Ala	Gly	TTA Leu	Phe 590	Ala	Gly	1776
55	Trp	Val	595	Gin	Ile	Val	Asn	Asp 600	Phe	Val	Ile	Glu	GCT Ala 605	Asn	Lys	Ser	1824
60	Asn	610	Met	Asp	Lys	Ile	Ala 615	Asp	Ile	Ser	Leu	11e 620	GTT Val	Pro	Tyr	Ile	1872
	Gly 625	Leu	Ala	Leu	Asn	Val 630	Gly	Asn	Glu	Thr	Ala 635	Lys		Asn	Phe	Glu 640	1920
65		Ala	Phe	Glu	11e 645	Ala	Gly	Ala	Ser	11e 650	Leu	Leu	Glu	Phe	Ile 655	Pro	1968
70	GAA Glu	CTT Leu	TTA Leu	ATA Ile	CCT Pro	GTA Val	GTT Val	GGA Gly	GCC Ala	TTT Phe	TTA Leu	TTA Leu	GAA Glu	TCA Ser	TAT Tyr	ATT Ile	2016

	•			66	0				66	5				67	0		
5			67	5	/-	, 110		680	0	. 116	: Asp) Ası	1 Ala 689	TT.	A AC u Th	T AAA r Lys	2064
10		69	0	1		, ,,,	695	5	- Iyı	Gly	Leu	700	e Val)	. Ala	a Gl	A TGG n Trp	2112
	709	5				710	J1.		- IYI	1111	715	Lys	Glu	Gly	/ Met	TAT Tyr 720	2160
15	AAC Lys	GCT Ala	TTA Lei	AAA Asr	TAT Tyr 725		GCA Ala	CAA Gln	GCA Ala	TTG Leu 730	GIU	GAA Glu	ATA Ile	ATA Ile	AAA Lys 735	A TAC Tyr	2208
20	AGA Arg	TAT	AAT Asn	TATA	TAT Tyr	TCT Ser	GAA Glu	AAA Lys	GAA Glu 745	Lys	TCA Ser	AAT Asn	ATT	AAC Asn 750	Ile	GAT Asp	2256
25			755			001	Lys	760	ASII	GIU	GIÀ	ile	765	Gln	Ala	`ATA Ile	2304
30	GAT Asp	AAT Asn 770	ATA Ile	AAT Asn	AAT Asn	TTT Phe	ATA Ile 775	AAT Asn	GGA Gly	TGT Cys	TCT Ser	GTA Val 780	TCA Ser	TAT Tyr	TTA Leu	ATG Met	2352
	AAA Lys 785	AAA Lys	ATG Met	ATT Ile	CCA Pro	TTA Leu 790	GCT Ala	GTA Val	GAA Glu	AAA Lys	TTA Leu 795	CTA Leu	GAC Asp	TTT Phe	GAT Asp	AAT Asn 800	2400
35	ACT Thr	CTC Leu '	AAA Lys	AAA Lys	AAT Asn 805	TTG Leu	TTA Leu	AAT Asn	TAT Tyr	ATA Ile 810	GAT Asp	GAA Glu	AAT Asn	AAA Lys	TTA Leu 815	TAT Tyr	2448
40	TTG Leu	ATT Ile	GGA Gly	AGT Ser 820	GCA Ala	GAA Glu	TAT Tyr	GAA Glu	AAA Lys 825	TCA Ser	AAA Lys	GTA Val	AAT Asn	AAA Lys 830	TAC Tyr	TTG Leu	2496
45	AAA Lys	ACC Thr	ATT Ile 835	ATG Met	CCG Pro	TTT Phe	GAT Asp	CTT Leu 840	TCA Ser	ATA Ile	TAT Tyr	ACC Thr	AAT Asn 845	GAT Asp	ACA Thr	ATA Ile	2544
50	CTA Leu	ATA Ile 850	GAA Glu	ATG Met	TTT Phe	AAT Asn	AAA Lys 855	TAT Tyr	AAT Asn	AGC Ser	GAA Glu	ATT Ile 860	TTA Leu	AAT Asn	AAT Asn	ATT Ile	2592
	ATC Ile 865	TTA Leu	AAT Asn	TTA Leu	AGA Arg	TAT Tyr 870	AAG Lys	GAT Asp	AAT Asn	ASN	TTA Leu 875	ATA Ile	GAT Asp	TTA Leu	TCA Ser	GGA Gly 880	2640
55	TAT Tyr	GGG Gly	GCA Ala	AAG Lys	GTA Val 885	GAG Glu	GTA Val	TAT Tyr	Asp	GGA Gly 890	GTC Val	GAG Glu	CTT Leu	AAT Asn	GAT Asp 895	AAA Lys	2688
60	AAT Asn	CAA Gln	TTT Phe	AAA Lys 900	TTA Leu	ACT . Thr	AGT Ser	TCA Ser	GCA Ala 905	AAT . Asn .	AGT . Ser	AAG Lys	ATT Ile	AGA Arg 910	GTG Val	ACT Thr	2736
65	CAA Gin	AAT Asn	CAG Gln 915	AAT Asn	ATC Ile	ATA 1	FIIE	AAT Asn 920	AGT Ser	GTG ' Val	TTC	Leu	GAT Asp 925	TTT Phe	AGC Ser	GTT Val	2784
70	AGC Ser	TTT Phe 930	TGG Trp	ATA Ile	AGA . Arg	ATA (Pro 935	AAA Lys	TAT . Tyr	AAG /	Asn A	GAT Asp 940	GGT . Gly	ATA Ile	CAA Gln	AAT Asn	2832

	TAT Tyr 945	TIG	CAT His	AAT Asn	GAA Glu	TAT Tyr 950	Thr	ATA Ile	ATT	AAT Asn	TGT Cys 955	Met	AAA Lys	AAT Asn	AAT Asn	TCG Ser 960	2880
5	GGC Gly	TGG Trp	AAA Lys	ATA Ile	TCT Ser 965	ATT Ile	AGG Arg	GGT Gly	AAT Asn	AGG Arg 970	Ile	ATA Ile	TGG Trp	ACT Thr	TTA Leu 975		2928
10	GAT Asp	ATA Ile	AAT Asn	GGA Gly 980	Lys	ACC Thr	AAA Lys	TCG Ser	GTA Val 985	Phe	TTT Phe	GAA Glu	TAT Tyr	AAC Asn 990	Ile	AGA Arg	2976
15	GAA Glu	GAT Asp	ATA Ile 995	TCA Ser	GAG Glu	TAT Tyr	ATA Ile	AAT Asn 100	Arg	TGG Trp	TTT Phe	TTT Phe	GTA Val 100	Thr	ATT	ACT Thr	3024
20	AAT Asn	AAT Asn 101	Leu	AAT Asn	AAC Asn	GCT Ala	AAA Lys 101	Ile	TAT Tyr	ATT Ile	AAT Asn	GGT Gly 1026	Lys	CTA Leu	GAA Glu	TCA Ser	3072
	AAT Asn 1029	Thr	GAT Asp	ATT Ile	AAA Lys	GAT Asp 1030	Ile	AGA Arg	GAA Glu	GTT Val	ATT Ile 1035	Ala	AAT Asn	GGT Gly	GAA Glu	ATA Ile 1040	3120
25	ATA Ile	TTT Phe	AAA Lys	TTA Leu	GAT Asp 1045	Gly	GAT Asp	ATA Ile	GAT Asp	AGA Arg 105	Thr	CAA Gln	TTT Phe	ATT Ile	TGG Trp 105	Met	3168
30	AAA Lys	TAT Tyr	TTC Phe	AGT Ser 106	ATT Ile O	TTT Phe	AAT Asn	ACG Thr	GAA Glu 1069	Leu	AGT Ser	CAA Gln	TCA Ser	AAT Asn 107	Ile	GAA Glu	3216
35	GAA Glu	AGA Arg	TAT Tyr 1075	Lys	ATT Ile	CAA Gln	TCA Ser	TAT Tyr 1080	Ser	GAA Glu	TAT Tyr	TTA Leu	AAA Lys 1089	Asp	TTT Phe	TGG Trp	3264
40	GGA Gly	AAT Asn 1090	Pro	TTA Leu	ATG Met	TAC Tyr	AAT Asn 1095	Lys	GAA Glu	TAT Tyr	TAT Tyr	ATG Met 1100	Phe	AAT Asn	GCG Ala	GGG Gly	3312
	AAT Asn 1105	Lys	AAT Asn	TCA Ser	TAT Tyr	ATT Ile 1110	Lys	CTA Leu	AAG Lys	AAA Lys	GAT Asp 1115	Ser	CCT Pro	GTA Val	GGT Gly	GAA Glu 1120	3360
45	ATT	TTA Leu	ACA Thr	CGT Arg	AGC Ser 1125	Lys	TAT Tyr	AAT Asn	CAA Gln	AAT Asn 1130	Ser	AAA Lys	TAT Tyr	ATA Ile	AAT Asn 1139	Tyr	3408
50	AGA Arg	GAT Asp	TTA Leu	TAT Tyr 1140	ATT Ile	GGA Gly	GAA Glu	AAA Lys	TTT Phe 1145	Ile	ATA Ile	AGA Arg	Arg	AAG Lys 1150	Ser	AAT Asn	3456
55	TCT Ser	CAA Gln	TCT Ser 1155	11e	AAT Asn	GAT Asp	GAT Asp	ATA Ile 1160	Val	AGA Arg	AAA Lys	GAA Glu	GAT Asp 1165	Tyr	ATA Ile	TAT Tyr	3504
60	CTA Leu	GAT Asp 1170	Pne	TTT Phe	AAT Asn	TTA Leu	AAT Asn 1175	CAA Gln	GAG Glu	TGG Trp	Arg	GTA Val 1180	Tyr	ACC Thr	TAT Tyr	AAA Lys	3552
	TAT Tyr 1185	rne	AAG Lys	AAA Lys	GAG Glu	GAA Glu 1190	Glu	AAA Lys	TTG Leu	TTT Phe	TTA Leu 1195	Ala	CCT Pro	ATA Ile	AGT Ser	GAT Asp 1200	3600
65	TCT Ser	GAT Asp	GAG Glu	TTT Phe	TAC Tyr 1205	Asn	ACT Thr	ATA Ile	CAA Gln	ATA Ile 1210	Lys	GAA Glu	TAT Tyr	GAT Asp	GAA Glu 1215	Gln	3648

	CCA Pro	ACA Thi	TAT	AGT Ser 122	. cys	CAG Gln	TTG Leu	CTI Let	TTT 1 Phe 122	; Lys	AAA Lys	GAT Asp	GAA	GA/	ي Se	T ACT		3696
5	GAT Asp	GAC Glu	3 ATA 1 Ile 123		TTG Leu	ATT Ile	GGT	ATT	HIS	CGT Arg	TTC Phe	TAC	GAA Glu 124	Sei	r GGA	A ATT		3744
10	GTA Val	TT1 Phe 125		GAG Glu	TAT	AAA Lys	GAT Asp 125	INT	TTT Phe	TGT Cys	ATA Ile	AGT Ser 126	Lys	TGC Trp	TAC Tyr	TTA Leu	,	3792
15	126	5		2,3	AL 9	127	0	ıyr	ASN	ren	Lys 127	Leu 5	Gly	TG1 Cys	AAT Asn	TGG Trp 1280		3840
20		rne	116	CCT Pro	128	Asp 5	GIu	Gly	Trp	ACT Thr 129	Glu	AAT						3876
	(2)	INF	ORMA	TION	FOR	SEQ	ID I	NO : 4	2:									
25'			(i)	(B	ENCE) LEI) TY) TO	NGTH PE: a	: 12: amino	91 a	mino id	: aci	ds							
		(ii)	MOLE	CULE	TYP	E: pi	rote	in									
30		(.	xi)	SEQU	ENCE	DES	CRIPT	rion	: SE	DI Ç	NO :	12:						
	Met 1	Pro	Val	Thr	Ile 5	Asn	Asn	Phe	Asn	Tyr 10	Asn	Asp	Pro	Ile	Asp 15	Asn		
35	Asn	Asn	Ile	Ile 20	Met	Met	Glu	Pro	Pro 25	Phe	Ala	Arg	Gly	Thr 30	Gly	Arg		
40	Tyr	Tyr	Lys 35	Ala	Phe	Lys	Ile	Thr 40	Asp	Arg	Ile	Trp	Ile 45	Ile	Pro	Glu		
	Arg	Tyr 50	Thr	Phe	Gly	Tyr	Lys 55	Pro	Glu	Asp	Phe	Asn 60	Lys	Ser	Ser	Gly		
45	Ile 65	Phe	Asn	Arg	Asp	Val 70	Cys	Glu	Tyr	туr	Asp 75	Pro	Asp	туг	Leu	Asn 80		
•	Thr	Asn	Asp	Lys	Lys 85	Asn	Ile	Phe	Leu	Gln 90	Thr	Met	Ile	Lys	Leu 95	Phe		
50	Asn	Arg	Ile	Lys 100	Ser	Lys	Pro	Leu	Gly 105	Glu	Lys	Leu	Leu	Glu 110	Met	Ile		
55	Ile	Asn	Gly 115	Ile	Pro	Tyr	Leu	Gly 120	Asp	Arg	Arg	Val	Pro 125	Leu	Glu	Glu		
	Phe	Asn 130	Thr	Asn	Ile	Ala	Ser 135	Val	Thr	Val	Asn	Lys 140	Leu	Ile	Ser	Asn		
60	Pro 145	Gly	Glu	Val	Glu	Arg 150	Lys	Lys	Gly	Ile	Phe 155	Ala	Asn	Leu	Ile	Ile 160		

•	Phe	Gly	Pro	Gly	Pro 165	Val	Leu	Asn	Glu	Asr 170	Glu	Thr	· Ile	Asp	11e	Gly
5	Ile	Gln	Asn	His 180	Phe	Ala	Ser	Arg	Glu 185		Phe	Gly	Gly	7 Ile 190		Gln
	Met	Lys	Phe 195	Cys	Pro	Glu	Tyr	Val 200	Ser	Val	Phe	Asn	Asn 205		Gln	Glu
10	Asn	Lys 210	Gly	Ala	Ser	Ile	Phe 215	Asn	Arg	Arg	Gly	Туг 220		Ser	Asp	Pro
15	Ala 225	Leu	Ile	Leu	Met	His 230	Glu	Leu	Ile	His	Val 235	Leu	His	Gly	Leu	Tyr 240
	Gly	Ile	Lys	Val	Asp 245	Asp	Leu	Pro	Ile	Val 250	Pro	Asn	Glu	Lys	Lys 255	
20	Phe	Met	Gln	Ser 260	Thr	Asp	Ala	Ile	Gln 265	Ala	Glu	Glu	Leu	Tyr 270	Thr	Phe
4.5	Gly	Gly	Gln 275	Asp	Pro	Ser	Ile	Ile 280	Thr	Pro	Ser	Thr	Asp 285	Lys	Ser	Ile
`2 5	Tyr	Asp 290	Lys	Val	Leu	Gln	Asn 295	Phe	Arg	Gly	Ile	Val 300	Asp	Arg	Leu	Asn
30	Lys 305	Val	Leu	Val	Cys	Ile 310	Ser	Asp	Pro	Asn	Ile 315	Asn	Ile	Asn	Ile	Tyr 320
		:			Lys 325					330					335	_
35				340	Asp				345					350		
4.0			355		Thr			360					365			-
40	Thr	Arg 370	Ala	Ser	Tyr	Phe	Ser 375	Asp	Ser	Leu	Pro	Pro 380	Val	Lys	Ile	Lys
45	385				Asn	390					395					400
					Met 405					410					415	
50				420	Tyr				425					430		
. .			435		Cys			440					445			_
55		450			Asp		455					460				
60	400				Lys	4/0					475					480
	Tyr	Ile	Glu	Asn	Asp 485	Phe	Pro	Ile	Asn	Glu 490	Leu	Ile	Leu	Asp	Thr 495	Asp
65	Leu			500					505					510		
30	Asp	Phe	Asn 515	Val	qzA	Val	Pro	Val 520	Tyr	Glu	Lys	Gln	Pro 525	Ala	Ile	Lys
70	Lys	Ile	Phe	Thr	Asp	Glu	Asn	Thr	Ile	Phe	Gln	Tyr	Leu	Tyr	Ser	Gln

		530					535	•				540)			
5	Thr 545	Phe	Leu	Leu	Asp	Ile 550	Arg	Asp	Ile	e Ser	Leu 555	Thr	Ser	Ser	Phe	Ası 560
					203					Tyr 570	!				575	
10				200					585					590)	
1.5			,,,					600		Val			605			
15							012			Ser		620				
20						030				Thr	635					640
					043					11e 650					655	
25				000					665	Phe				670		
30			0,3					980		Ile			685			
.50		000					ככס			Gly		700				
35	. 4.3					/10				Thr	715					720
					723					Leu 730					735	
40	Arg			740					/45					750		
45			, , ,					/6 U		Glu			765			
4.,		,,,					//5					780				
50	Lys 785					790					795					800
	Thr				805					810					815	
55	i.eu			020					825					830		
	Lys		933					840					845			
60		030					855					860				
65	11e :					670					875					880
	Tyr				003					890					895	
70	Asn (Gln	Phe	Lys 900	Leu	Thr	Ser	Ser	Ala 905	Asn	Ser	Lys	Ile	Arg 910	Val	Thr

ı	Gl	n Ası	915	Asr	ı Ile	e Ile	e Ph	e Ası 920	n Ser	· Va	l Phe	e Leu	Asp 925	Phe	Ser	val
5	Se	930	e Trp) Ile	e Arg	Ile	93	o Lys 5	5 Tyr	Ly	s Asn	Asp 940	Gly	Ile	Glr	Asn
	Ту: 94	r Ile	His	Asn	Glu	Tyr 950	Thi	r Ile	e Ile	: Ası	n Cys 955	Met	Lys	' Asn	Asn	Ser 960
10	Glγ	/ Trp	Lys	Ile	Ser 965	Ile	Arg	g Gly	/ Asn	970	g Ile	lle	Trp	Thr	Leu 975	
15	Asp) Ile	: Asn	Gly 980	Lys	Thr	Lys	s Ser	Val 985	Phe	Phe	Glu	Tyr	Asn 990	Île	Arg
	Glu	Asp	11e 995	Ser	Glu	Tyr	Ilε	Asn 100	Arg	Trp	Phe	Phe	Val 100		Ile	Thr
20	Asn	Asn 101	Leu 0	Asn	Asn	Ala	Lys 101	Ile 5	Tyr	Ιlε	: Asn	Gly 102	Lys	Leu	Glu	Ser
	Λsn 102	Thr 5	Asp	Ile	Lys	Asp 103	Ile O	Arg	Glu	Val	Ile 103	Ala 5	Asn	Gly	Glu	Ile 1040
125					104:	•				105					105	5
30				1000					1065	5	Ser			1070)	
								1081	U		Tyr		1085			
35		10,0					109	5			Tyr	1100	1			
40						1110	,				Asp 1115	5				1120
40					1123					113					1135	
45				1140	,				1145		Ile			1150		
								1160	,	•	Lys		1165			
50							11/5	•				1180				
55						1130					Leu 1195					1200
					1205					1210				1	215	
60				1220					1225		Lys :		1	.230		
			***					1240			Phe '	3	1245			
55						•	1235					1260				
7()						2/0					Lys 1 1275	Leu C	Sly C	ys A		Trp 1280
	Gln	ene .	rie p	-10 I	∟ys Æ	sp (ilu	Gly :	rrp 1	Chr -	Glu					

1285 1290

	,	2111	OKINA	TION	FOR	SEQ	10	NU: 4	3 :								
5		(i	(QUEN A) L B) T C) S D) T	ENGT YPE : TRAN	H: 1 nuc DEDN	526 leic ESS:	base aci dou	pai d	rs							
10		(ii) MO	LECU A) D	LE T ESCR	YPE: IPTI	oth ON:	er n /des	ucle C =	ic a "DNA	cid "						
15		(ix	(.	ATUR A) N B) L	AME/			15	23								
		(xi) SE	QUEN	CE D	ESCR	IPTI	ON:	SEQ	ID N	0:43	:					
20	AGA'	TCTC	GAT	CCCG	CGAA	AT T	AATA	CGAC'	T CA	CTAT.	AGGG	GAA	TTGT	GAG	CGGA'	TAACAA	60
25	TTC	CCCT	CTA (GAAA'	TAAT'	TT T	GTTT.	AACT"	T TA	AGAA	GGAG	ATA	TACC		GGC Gly		116
	CAT His	CAT His 5	CAT His	CAT His	CAT His	CAT His	CAT His 10	CAT His	CAC His	AGC Ser	AGC Ser	GGC Gly 15	CAT His	ATC Ile	GAA Glu	GGT Gly	164
30	CGT Arg 20	CAT His	ATG Met	GCT Ala	AGC Ser	ATG Met 25	GCT Ala	GAT Asp	ACA Thr	ATA Ile	CTA Leu 30	ATA Ile	GAA Glu	ATG Met	TTT Phe	AAT Asn 35	212
35	ΛΑΑ Lys	TAT Tyr	AAT Asn	AGC Ser	GAA Glu 40	ATT Ile	TTA Leu	AAT Asn	AAT Asn	ATT Ile 45	ATC Ile	TTA Leu	AAT Asn	TTA Leu	AGA Arg 50	TAT Tyr	260
4 0	AGA Arg	GAT Asp	AAT Asn	AAT Asn 55	TTA Leu	ATA Ile	GAT Asp	TTA Leu	TCA Ser 60	GGA Gly	TAT Tyr	GGA Gly	GCA Ala	AAG Lys 65	GTA Val	GAG Glu	308
4 5	GTA Val	TAT Tyr	GAT Asp 70	GGG Gly	GTC Val	AAG Lys	CTT Leu	AAT Asn 75	GAT Asp	AAA Lys	AAT Asn	CAA Gln	TTT Phe 80	AAA Lys	TTA Leu	ACT Thr	356
	AGT Ser	TCA Ser 85	GCA Ala	GAT Asp	AGT Ser	AAG Lys	ATT Ile 90	AGA Arg	GTC Val	ACT Thr	CAA Gln	AAT Asn 95	CAG Gln	AAT Asn	ATT	ATA Ile	404
5()	TTT Phe 100	AAT Asn	AGT Ser	ATG Met	TTC Phe	CTT Leu 105	GAT Asp	TTT Phe	AGC Ser	GT T Val	AGC Ser 110	TTT Phe	TGG Trp	ATA Ile	AGG Arg	ATA Ile 115	452
55	CCT Pro	AAA Lys	TAT Tyr	AGG Arg	AAT Asn 120	GAT Asp	GAT Asp	ATA Ile	CAA Gln	AAT Asn 125	TAT Tyr	ATT Ile	CAT His	AAT Asn	GAA Glu 130	TAT Tyr	500
5()	ACG Thr	ATA Ile	ATT Ile	AAT Asn 135	TGT Cys	ATG Met	AAA Lys	AAT Asn	AAT Asn 140	TCA Ser	GGC Gly	TGG Trp	AAA Lys	ATA Ile 145	TCT Ser	ATT Ile	548
55	AGG Arg	GGT Gly	AAT Asn 150	AGG Arg	ATA Ile	ATA Ile	TGG Trp	ACC Thr 155	TTA Leu	ATT Ile	GAT Asp	ATA Ile	AAT Asn 160	GGA Gly	AAA Lys	ACC Thr	596
,	AAA Lys	TCA Ser 165	GTA Val	TTT Phe	TTT Phe	GAA Glu	TAT Tyr 170	AAC Asn	ATA Ile	AGA Arg	GAA Glu	GAT Asp 175	ATA Ile	TCA Ser	GAG Glu	TAT Tyr	644
7()	ATA	AAT	AGA	TGG	TTT	T TT	GTA	ACT	ATT	ACT	AAT	TAA	TTG	GAT	AAT	GCT	692

	Ile 180	Asn	Arg	Trp	Phe	Phe 185	Val	Thr	: Ile	Thr	Asn 190		Leu	Asp	Asr	1 Ala 195	
5	AAA Lys	ATT	TAT Tyr	ATT	AAT Asn 200	Gly	ACG Thr	TTA Leu	GAA Glu	TCA Ser 205	Asn	ATG Met	GAT Asp	ATI	AAA Lys 210	GAT Asp	740
10	ATA Ile	GGA Gly	GAA Glu	GTT Val 215	Ile	GTT Val	AAT Asn	GGT Gly	GAA Glu 220	Ile	ACA Thr	TTT Phe	AAA Lys	TTA Leu 225	Asp	GGT Gly	788
15	GAT Asp	GTA Val	GAT Asp 230	Arg	ACA Thr	CAA Gln	TTT Phe	ATT Ile 235	Trp	ATG Met	AAA Lys	TAT	TTT Phe 240	Ser	ATT	TTT	836
	AAT Asn	ACG Thr 245	CAA Gln	TTA Leu	AAT Asn	CAA Gln	TCA Ser 250	AAT Asn	ATT Ile	AAA Lys	GAG Glu	ATA Ile 255	TAT Tyr	AAA Lys	ATT	CAA Gln	884
20	TCA Ser 260	TAT Tyr	AGC Ser	GAA Glu	TAC Tyr	TTA Leu 265	AAA Lys	GAT Asp	TTT Phe	TGG Trp	GGA G1y 270	Asn	CCT Pro	TTA Leu	ATG Met	TAT Tyr 275	932
25	AAT Asn	AAA Lys	GAA Glu	TAT Tyr	TAT Tyr 280	ATG Met	TTT Phe	AAT Asn	GCG Ala	GGG Gly 285	AAT Asn	AAA Lys	AAT Asn	TCA Ser	TAT Tyr 290	ATT Ile	980
30	AAA Lys	CTA Leu	GTG Val	AAA Lys 295	GAT Asp	TCA Ser	TCT Ser	GTA Val	GGT Gly 300	GAA Glu	ATA Ile	TTA Leu	ATA Ile	CGT Arg 305	AGC Ser	AAA Lys	1028
35	TAT Tyr	AAT Asn	CAG Gln 310	AAT Asn	TCC Ser	AAT Asn	TAT Tyr	ATA Ile 315	AAT Asn	TAT Tyr	AGA Arg	AAT Asn	TTA Leu 320	TAT Tyr	ATT Ile	GGA Gly	1076
	GAA Glu	AAA Lys 325	TTT Phe	ATT Ile	ATA Ile	AGA Arg	AGA Arg 330	GAG Glu	TCA Ser	AAT Asn	TCT Ser	CAA Gln 335	TCT Ser	ATA Ile	AAT Asn	GAT Asp	1124
40	GAT Asp 340	ATA Ile	GTT Val	AGA Arg	AAA Lys	GAA Glu 345	GAT Asp	TAT Tyr	ATA Ile	CAT His	CTA Leu 350	GAT Asp	TTG Leu	GTA Val	CTT Leu	CAC His 355	1172
45	CAT His	GAA Glu	GAG Glu	TGG Trp	AGA Arg 360	GTA Val	TAT Tyr	GCC Ala	TAT Tyr	AAA Lys 365	TAT Tyr	TTT Phe	AAG Lys	GAA Glu	CAG Gln 370	GAA Glu	1220
50	GAA Glu	AAA Lys	TTG Leu	TTT Phe 375	TTA Leu	TCT Ser	ATT Ile	ATA Ile	AGT Ser 380	GAT Asp	TCT Ser	AAT Asn	GAA Glu	TTT Phe 385	TAT Tyr	AAG Lys	1268
55	ACT Thr	ATA Ile	GAA Glu 390	ATA Ile	AAA Lys	GAA Glu	TAT Tyr	GAT Asp 395	GAA Glu	CAG Gln	CCA Pro	TCA Ser	TAT Tyr 400	AGT Ser	TGT Cys	CAG Gln	1316
	TTG Leu	CTT Leu 405	TTT Phe	AAA Lys	AAA Lys	GAT Asp	GAA Glu 410	GAA Glu	AGT Ser	ACT Thr	GAT Asp	GAT Asp 415	ATA Ile	GGA Gly	TTG Leu	ATT	1364
60	GGT Gly 420	ATT Ile	CAT Hìs	CGT Arg	TTC Phe	TAC Tyr 425	GAA Glu	TCT Ser	GGA Gly	GTT Val	TTA Leu 430	CGT Arg	AAA Lys	AAG Lys	TAT Tyr	AAA Lys 435	1412
65	GAT Asp	TAT Tyr	TTT Phe	TGT Cys	ATA Ile 440	AGT Ser	AAA Lys	TGG Trp	TAC Tyr	TTA Leu 445	AAA Lys	GAG Glu	GTA Val	AAA Lys	AGG Arg 450	AAA Lys	1460
70	CCA Pro	TAT Tyr	AAG Lys	TCA Ser 455	AAT Asn	TTG Leu	GGA Gly	TG T Cys	AAT Asn 460	TGG Trp	CAG Gln	TTT Phe	TTA 1le	CCT Pro 465	AAA Lys	GAT Asp	1508

GAA GGG TGG ACT GAA TAA Glu Gly Trp Thr Glu 470

			4 / 0													
5	(2)	INF	orma	TION	FOR	SEQ	ID	NO : 4	4:							
10			(i)	(A (B	ENCE) LE) TY) TO	NGTH PE :	: 47 amin	2 am o ac	ino id	: acid	s					
		(ii)	MOLE	CULE	TYP	E: p	rote	in							
15		(xi)	SEQU	ENCE	DES	CRIP	TION	: SE	Q ID	NO:	44:				
	Met 1	Gly	His	His	His 5	His	Hıs	His	His	His 10	Hıs	His	Ser	Ser	Gly 15	His
,20	Ile	Glu	Gly	Arg 20	His	Met	Ala	Ser	Met 25	Ala	Asp	Thr	Ile	Leu 30	Ile	Glu
	Met	Phe	Asn 35	Lys	Tyr	Asn	Ser	Glu 40	Ile	Leu	Asn	Asn	Ile 45	Ile	Leu	Asn
25	Leu	Arg 50	Tyr	Arg	Asp	Asn	Asn 55	Leu	Ile	Asp	Leu	Ser 60	Gly	Tyr	Gly	Ala
30	Lys 65	Val	Glu	Val	Tyr	Asp 70	Gly	Val	Lys	Leu	Asn 75	Asp	Lys	Asn	Gln	Phe 80
	Lys	Leu	Thr	Ser	Ser 85	Λla	Asp	Ser	Lys	Ile 90	Arg	Val	Thr	Gln	Asn 95	Gln
35	Asn	Ile	lie	Phe 100	Asn	Ser	Met	Phe	Leu 105	Asp	Phe	Ser	Val	Ser 110	Phe	Trp
	lle	Arg	11e 115	Pro	Lys	Туг	Arg	Asn 120	Asp	Asp	Ile	Gln	Asn 125	Tyr	Ile	His
40	Asn	Glu 130	Тут	Thr	Ile	Ile	Asn 135	Cys	Met	Lys	Asn	Asn 140	Ser	Gly	Trp	Lys
45	lle 145	Ser	Ile	Arg	Gly	Asn 150	Arg	Ile	Ile	Trp	Thr 155	Leu	Ile	Asp	Ile	Asn 160
	Gly	L;;s	Thr	Lys	Ser 165	Val	Phe	Phe	Glu	Tyr 170	Asn	Iie	Arg	Glu	Asp 175	Ile
50	Ser	Glu	Tyr	Ile 180	Asn	Arg	Trp	Phe	Phe 185	Val	Thr	ſle	Thr	Asn 190	Asn	Leu
			195					200					Ser 205			
55	Ile	Lys 210	Asp	lle	Gly	Glu	Val 215	Ile	Val	Asn	Gly	Glu 220	Ile	Thr	Phe	Lys
60	Leu 225	Asp	Gly	Asp	Val	Asp 230	Arg	Thr	Gln	Phe	11e 235	Trp	Met	Lys	Tyr	Phe 240
	Ser	lle	Phe	Asn	Thr 245	Gln	Leu	Asn	Gln	Ser 250	Asn	Ile	Lys	Glu	Ile 255	Tyr
65	Lys	Ile	Gln	Ser 260	Туг	Ser	Glu	Tyr	Leu 265	Lys	Asp	Phe	Trp	Gly 270	Asn	Pro
	Leu	Met	Tyr 275	Asn	Lys	Glu	Tyr	Tyr 280	Met	Phe	Asn	Ala	Gly 285	Asn	Lys	Asn
70	Ser	Tyr	Ile	Lys	Leu	Val	Lys	Asp	Ser	Ser	Val	Gly	Glu	ıle	Leu	Ile

- 302 -

		290					295					300					
5	Arg 305	Ser	Lys	Tyr	Asn	Gln 310	Asn	Ser	Asn	Tyr	Ile 315	Asn	Tyr	Arg	Asn	Leu 320	
	Tyr	Ile	Gly	Glu	Lys 325	Phe	Ile	Ile	Arg	Arg 330	Glu	Ser	Asn	Ser	Gln 335	Ser	
10	Ile	Asn	Asp	Asp 340	Ile	Val	Arg	Lys	Glu 345	Asp	Tyr	Ile	His	Leu 350	Asp	Leu	
	Val	Leu	His 355	His	Glu	Glu	Trp	Arg 360	Val	Tyr	Ala	Tyr	Lys 365	Tyr	Phe	Lys	
15	Glu	Gln 370	Glu	Glu	Lys	Leu	Phe 375	Leu	Ser	Ile	Ile	Ser 380	Asp	Ser	Asn	Glu	
20	Phe 385	Tyr	Lys	Thr	Ile	Glu 390	Ile	Lys	Glu	Tyr	Asp 395	Glu	Gln	Pro	Ser	Tyr 400	
	Ser	Cys	Gln	Leu	Leu 405	Phe	Lys	Lys	Asp	Glu 410	Glu	Ser	Thr	Asp	Asp 415	Ile	
25	Gly	Leu	Ile	Gly 420	Ile	His	Arg	Phe	Tyr 425	Glu	Ser	Gly	Val	Leu 430	Arg	Lys	
	Lys	Tyr	Lys 435	Asp	Tyr	Phe	Cys	Ile 440	Ser	Lys	Trp	Tyr	Leu 445	Lys	Glu	Val	
30	Lys	Arg 450	Lys	Pro	Tyr	Lys	Ser 455	Asn	Leu	Gly	Cys	Asn 460	Trp	Gln	Phe	Ile	
35	Pro 465	Lys	Asp	Glu	Gly	Trp 470	Thr	Glu									
- //-	(2)		RMAT														
40		(1)	(E	L) LE L) TY L) ST	EE CH ENGTH (PE: PANE POLC	I: 15 nucl EDNE	47 b eic SS:	ase acid doub	pair i	S							
45			MOL			PE:	DNA	(ger	omic	:)							
1		(1%)) NA	ME/K CATI			. 152	:3								
50		(xi)	SEQ	UENC	E DE	SCRI	PTIC	N: S	EQ I	D NC	:45:						
	AGAT	CTCG	SAT C	CCGC	GAAA	T TA	ATAC	GACT	CAC	TATA	.GGG	GAAT	TGTG	AG C	GGAT	'AACAA	60
55	TTC	CCTC	TA G	TAAA	TTAAT	T TG	TTTA	ACTT	' ТАА	.GAAG	GAG	АТАТ			GGC Gly		116
6()	CAT His	CAT His 5	CAT His	CAT His	CAT His	CAT His	CAT His 10	CAT His	CAC His	AGC Ser	AGC Ser	GGC Gly 15	CAT His	ATC Ile	GAA Glu	GGT Gly	164
65	CGT Arg 20	CAT His	ATG Met	GCT Ala	AGC Ser	ATG Met 25	GCT Ala	GAT Asp	ACA Thr	ATA Ile	CTA Leu 30	ATA Ile	GAA Glu	λTG Met	TTT Phe	AAT Asn 35	212
-	AAA Lys	TAT Tyr	AAT Asn	AGC Ser	GAA Glu 40	ATT Ile	TTA Leu	AAT Asn	AAT Asn	ATT Ile 45	ATC Ile	TTA Leu	AAT Asn	TTA Leu	AGA Arg 50	TAT Tyr	260
70	AAG	GAT	TAA	AAT	TTA	ATA	GAT	ATT	TCA	GGA	TAT	GGG	GCA	AAG	GTA	GAG	308

- 303 -

	Ly	s Ası	ı z A q	n Ası	Lei	ılle	: Asp) Let	ser 60	Gly	туз	Gly	/ Ala	Lys 65		l Glu	
5	GT: Va	A TAT	GA: C Asp	r GGA D Gly	A GT(GAG Glu	CTI Leu	AAT Asn 75	. Ash	AAA Lys	AA7 Asr	CAA Gln	TTT Phe	Lys	TT/	A ACT	· 356
10	AG: Sei	TCA Ser 89	A GCA Ala	AAT Asr	AGT Ser	AAG Lys	ATT Ile 90	Ary	GTG Val	ACT Thr	CAA Gln	AAT Asn 95	Gln	AAT Asr	T ATO	ATA : Ile	404
15	100)		_	- 13-0	105		rne	261	vaı	110	Phe	Trp	Ile	Arg	ATA Ile 115	452
0.00		•	•	-7-	120	·p	Ory	116	GIII	125	Tyr	ile	His	Asn	Glu 130		500
20				135	-,-		2,5	nan	140	Sei	GIY	Trp	Lys	Ile 145	Ser	ATT Ile	548
25		•	150	5			•••	155	Leu	11e	Asp	He	Asn 160	Gly	Lys		596
30	-	165		TTT Phe	• •••	Jiu	170	Maii	116	Arg	GIU	175	Ile	Ser	Glu	Tyr	644
35	180		,	TGG Trp		185	Val	1111	116	inr	190	Asn	Leu	Asn	Asn	Ala 195	692
•	•		•	ATT Ile	200	01,	13 y 3	Leu	GIU	205	Asn	Thr	Asp	Ile	Lys 210	Asp	740
40		J		GTT Val 215	-10	AIG	ASII	GLY	220	116	IIe	Phe	Lys	Leu 225	Asp	Gly	788
45			230	AGA Arg	1111	GIII	PHE	235	Trp	Met	Lys	Tyr	Phe 240	Ser	Ile	Phe	836
50		245	514	TTA Leu	361	GIII	250	ASN	116	Glu	Glu	Arg 255	Tyr	Lys	Ile	Gln	884
55	260	- 7 -		GAA Glu	171	265	пуз	Asp	Pne	Trp	G1y 270	Asn	Pro	Leu	Met	Tyr 275	932
	TAA neA	AAA Lys	GAA Glu	TAT Tyr	TAT Tyr 280	ATG Met	TTT Phe	AAT Asn	Ala	GGG Gly 285	AAT Asn	AAA Lys	AAT Asn	TCA Ser	TAT Tyr 290	ATT Ile	980
60	AAA Lys	CTA Leu	AAG Lys	AAA Lys 295	GAT Asp	TCA Ser	CCT Pro	vai	GGT Gly 300	GAA Glu	ATT Ile	TTA . Leu	Thr	CGT Arg 305	AGC Ser	AAA Lys	1028
65	TAT Tyr	AAT Asn	CAA Gln 310	AAT Asn	TCT Ser	AAA ' Lys '	ıyı	ATA Ile 315	AAT Asn	TAT . Tyr .	AGA Arg	Asp :	TTA Leu 320	TAT Tyr	ATT Ile	GGA Gly	1076

	GAA Glu	Lys 325	Pne	ATT	'ATA	AGA Arg	AGA Arg 330	A AG Lys	TCA Ser	AAT Asn	TCT Ser	CAA Gln 335	Sea	T ATA	AAT Ran	GAT Asp	112	4
5	GAT Asp 340	T T 6	GTT Val	AGA Arg	AAA Lys	GAA Glu 345	GAT Asp	TAT	ATA Ile	TAT Tyr	CTA Leu 350	Asp	TTT Phe	TTT Phe	AAT Asn	TTA Leu 355	117	2
10	ASII	GIII	Glu	Trp	360	vaı	Tyr	Thr	Tyr	Lys 365	Tyr	Phe	Lys	Lys	Glu 370		1220)
15	O1u	Буѕ	Leu	375	Leu	Ala	Pro	lie	Ser 380	Asp	Ser	Asp	Glu	Phe 385	Tyr	AAT Asn	1268	3
20		116	390	116	Lys	GAA Glu	Tyr	395	Glu	Gln	Pro	Thr	Tyr 400	Ser	Cys	Gln	1316	;
	TTG Leu	CTT Leu 405	TTT Phe	AAA Lys	AAA Lys	GAT Asp	GAA Glu 410	GAA Glu	AGT Ser	ACT Thr	GAT Asp	GAG Glu 415	ATA Ile	GGA Gly	TTG Leu	ATT Ile	1364	
25	GGT Gly 420	ATT Ile	CAT	CGT Arg	TTC Phe	TAC Tyr 425	GAA Glu	TCT Ser	GGA Gly	ATT Ile	GTA Val 430	TTT Phe	GAA Glu	GAG Glu	TAT Tyr	AAA Lys 435	1412	
30	GAT Asp	TAT Tyr	TTT Phe	TGT Cys	ATA Ile 440	AGT Ser	AAA Lys	TGG Trp	TAC Tyr	TTA Leu 445	AAA Lys	GAG Glu	GTA Val	AAA Lys	AGG Arg 450	AAA Lys	1460	
35	CCA Pro	TAT Tyr	AAT Asn	TTA Leu 455	AAA Lys	TTG Leu	GGA Gly	TGT Cys	AAT Asn 460	TGG Trp	CAG Gln	TTT Phe	ATT Ile	CCT Pro 465	AAA Lys	GAT Aap	1508	
40	GAA Glu	GGG Gly	TGG Trp 470	ACT Thr	GAA Glu	TAAA	AGCT	TG C	GGCC	GCAC	T CG	AG					1547	
	(2)	INFO	DRMAT	'ION	FOR	SEQ	ID N	0:46	i :									
45		((i) S	(A)	LEN TYP	CHAR IGTH: E: a OLOG	472 mino	ami aci	.no a .d	cids								
		(i	.i) M	OLEC	ULE	TYPE	: pr	otei	n									
50		(×	i) S	EQUE	NCE	DESC	RIPT	ION:	SEQ	ID	NO : 4	6 :						
	Met 1	Gly	His	His	His 5	His :	His 1	His	His	His 10	His	His	Ser	Ser	Gly 15	His		
55	Ile	Glu	Gly	Arg 20	His	Met .	Ala :	Ser	Met . 25	Ala .	Asp '	Thr	lle	Leu 30	Ile	Glu		
60	Met	Phe	Asn 35	Lys	Tyr	Asn :	Ser (Glu 40	Ile	Leu .	Asn ,	Asn	Ile 45	Ile	Leu	Asn		
	Leu .	Arg 50	Tyr	Lys	Asp	Asn i	Asn 1 55	Leu	Ile A	Asp :	Leu :	Ser 60	Gly	Tyr (Gly	Ala		
65	Lys '	Va1	Glu	Val	Tyr .	Asp (Gly V	Jal (Glu 1	Leu i	Asn 7	Asp	Lys	Asn (Gln	Phe 80		
	Lys	Leu	Thr	Ser	Ser 85	Ala /	Asn S	Ser :	Lys :	lle 1 90	Arg V	Val '	Thr	Gln /	Asn (Gln		
70	Asn :	Ile	Ile	Phe .	Asn :	Ser \	/al E	Phe I	Leu /	Asp I	Phe s	Ser '	Val	Ser 1	Phe '	Trp		

				100					105					110		
5	Ile	Arg	Ile 115	Pro	Lys	Tyr	Lys	Asn 120	Asp	Gly	Ile	Gln	Asn 125	Tyr	Ile	His
•	Asn	Glu 130	Tyr	Thr	Ile	Ile	Asn 135	Cys	Met	Lys	Asn	Asn 140	Ser	GLy	Trp	Lys
10	143				Gly	150					155					160
15					Ser 165					170					175	
15				100	Asn				185					190		
20			195		Ile			200					205			
		210			Arg		215					220				
25	225				Ile	230					235					240
30					Thr 245					250					255	
				260	Tyr Lys				265					270		
35			2/5		Leu			280					285			
		290			Asn		295					300				
4()	305				Lys	310					315					320
45					325 Ile					330					335	
			Leu	340	Gln				345					350		
50		Glu	355		Lys		Phe	360					365			
55		370			Ile	Gln	3/5				Asp	380				
		Cys	Gln	Leu	Leu	390 Phe	Lys	Lys	Asp		395 Glu	Ser	Thr	Asp		400 Ile
60	Gly	Leu	lle	Gly 420	405 Ile	His	Arg	Phe	Tyr	410 Glu	Ser	Gly	Ile		415 Phe	Glu
	Glu	Tyr	Lys 435		Tyr	Phe	Cys	Ile	425 Ser	Lys	Trp	Tyr		430 Lys	Glu	Val
65	Lys	Arg 450		Pro	туr	Asn	Leu 455	440 Lys	Leu	Gly	Cys		445 Trp	Gln	Phe	Ile
70	Pro 465		Asp	Glu	Gly			Glu				460				
						470										

	(2)	INI	FORM	ATIO	V FO	R SE	Q ID	NO:	47:									
5		(i		(A) 1 (B) 1 (C) 1	LENG: TYPE STRAI	TH: : nuc NDEDI	ACTE 31 ba cleio NESS : lir	ase p c ac: : si:	pair: id	5							•	
10		(ii	.) MC	OLECT	JLE T	TYPE:	oth	ner i /des	nucle	eic a "DNA	acid A"							
		(xi) SE	EQUE	ICE I	DESCR	RIPTI	ON:	SEQ	ID 1	NO : 4	7 :						
15	CGC	CATG	GCT	GATA	CAA1	rac 1	TAAT	AGAA/	AT G									31
	(2)	INF	ORMA	OITA	FOF	SEC) ID	NO : 4	18:									
20	•	(i	((A) I (B) T (C) S	ENGT YPE : TRAN	TH: 2 nuc IDEDN	CTER 9 ba :leic !ESS:	se p aci sir	airs d	3								
25		(ii) MC	LECU (A) [LE T	YPE:	oth ON:	er n /des	ucle c =	ic a	cid							
		(xi) SE	QUEN	CE D	ESCR	IPTI	ON:	SEQ	ID N	10 : 48	1:						
30	GCA/	AGCT	TTT	ATTO	AGTO	CA C	CCTT	CATC	·									29
	(2)	INF	ORMA	TION	FOR	SEQ	ID	NO : 4	9:									
35		(i	. (A) L B) T C) S	ENGT YPE : TRAN	H: 3 nuc DEDN	CTER 753 leic ESS: lin	base aci dou	pai d	rs								
40		(ii) MO	LECU	LE T	YPE:	DNA	(ge	nomi	c)								
		(iz	€.		AME/	KEY: ION:	CDS	3750										
45		(xi)	SE	QUEN	CE D	ESCR	IPTI	ON:	SEQ	ID N	0:49	:						
	Met	CCA Pro	ACA Thr	ATT Ile	AAT Asn	AGT Ser	TTT Phe	AAT Asn	TAT Tyr	AAT Asn	GAT Asp	CCT Pro	GTT Val	AAT Asn	AAT Asn	AGA Arq		48
50	1				5					10					15	-		
	Thr	Ile	TTA Leu	TAT Tyr 20	ATT Ile	AAA Lys	CCA Pro	GGC Gly	GGT Gly 25	TGT Cys	CAA Gln	CAA Gln	TTT Phe	TAT Tyr 30	AAA Lys	TCA Ser		96
55	TTT Phe	AAT Asn	ATT Ile 35	ATG Met	AAA Lys	AAT Asn	ATT Ile	TGG Trp 40	ATA Ile	ATT Ile	CCA Pro	GAG Glu	AGA Arg 45	AAT Asn	GTA Val	ATT Ile	1	L44
60	GGT Gly	ACA Thr 50	ATT Ile	CCC Pro	CAA Gln	GAT Asp	TTT Phe 55	CTT Leu	CCG Pro	CCT Pro	ACT Thr	TCA Ser 60	TTG Leu	AAA Lys	AAT Asn	GGA Gly	1	192
65	GAT Asp 65	AGT Ser	AGT Ser	TAT Tyr	TAT Tyr	GAC Asp 70	CCT Pro	AAT Asn	TAT Tyr	TTA Leu	CAA Gln 75	AGT Ser	GAT Asp	CAA Gln	GAA Glu	AAG Lys 80	2	40
70	GAT . Asp	AAA Lys	TTT Phe	TTA Leu	AAA Lys 85	ATA Ile	GTC Val	ACA Thr	AAA Lys	ATA Ile 90	TTT Phe	AAT Asn	AGA Arg	ATA Ile	AAT Asn 95	GAT Asp	2	88

	AAT Asn	CTT Leu	TCA Ser	GGA Gly 100	Arg	ATT Ile	TTA Leu	TTA Leu	GAA Glu 105	GAA Glu	CTG Leu	TCA Ser	AAA Lys	GCT Ala 110	AAT Asn	CCA Pro	•	336
5	TAT Tyr	TTA Leu	GGA Gly 115	AAT Asn	GAT Asp	AAT Asn	ACT Thr	CCA Pro 120	GAT Asp	GGT Gly	GAC Asp	TTC Phe	ATT Ile 125	ATT	AAT Asn	GAT Asp		384
10	GCA Ala	TCA Ser 130	GCA Ala	GTT Val	CCA Pro	ATT Ile	CAA Gln 135	TTC Phe	TCA Ser	AAT Asn	GGT Gly	AGC Ser 140	CAA Gln	AGC Ser	ATA Ile	CTA Leu		432
15	145	110	ASII	vaı	me	11e 150	Met	GIY	Ala	Glu	Pro 155	Asp	TTA Leu	Phe	Glu	Thr 160		480
20	7.511	501	Ser	ASII	165	ser	reu	Arg	Asn	170	Tyr	Met	CCA Pro	Ser	Asn 175	His		528
2.5	GIY	FILE	GIY	180	iie	Ala	ile	Val	Thr 185	Phe	Ser	Pro	GAA Glu	Tyr 190	Ser	Phe		576
25	Arg	PHE	195	Asp	Asn	Ser	Met	200	Glu	Phe	Ile	Gln	GAT Asp 205	Pro	Ala	Leu	(624
30	1111	210	mec	HIS	GIU	Leu	11e 215	His	Ser	Leu	His	Gly 220	CTA Leu	Tyr	Gly	Ala	•	672
35	225	GLY	ire	Inr	inr	230	Tyr	Thr	Ile	Thr	Gln 235	Lys	CAA Gln	Asn	Pro	Leu 240		720
40	ΛΤΑ Ile	ACA Thr	AAT Asn	ATA Ile	AGA Arg 245	GGT Gly	ACA Thr	AAT Asn	ATT Ile	GAA Glu 250	GAA Glu	TTC Phe	TTA Leu	ACT Thr	TTT Phe 255	GGA Gly	•	768
	GGT Gly	ınr	Asp	260	Asn	Ile	Ile	Thr	Ser 265	Ala	Gln	Ser	Asn	Asp 270	Ile	Tyr		316
45	ACT Thr	ASII	275	Leu	Ala	Asp	Tyr	Lys 280	Lys	Ile	Ala	Ser	Lys 285	Leu	Ser	Lys	6	364
50		290	vai	Ser	Asn	Pro	Leu 295	Leu	Asn	Pro	Tyr	Lys 300	Asp	Val	Phe	Glu	9	912
55	GCA Ala 305	Lys	Tyr	GIY	Leu	310	Lys	Asp	Ala	Ser	Gly 315	lle	Tyr	Ser	Val	Asn 320	ġ	960
60	ATA Ile	AAC Asn	AAA Lys	TTT Phe	AAT Asn 325	GAT Asp	ATT Ile	TTT Phe	AAA Lys	AAA Lys 330	TTA Leu	TAC Tyr	AGC Ser	TTT Phe	ACG Thr 335	GAA Glu	10	800
	TTT Phe	GAT Asp	TTA Leu	GCA Ala 340	ACT Thr	AAA Lys	TTT Phe	CAA Gln	GTT Val 345	AAA Lys	TĠT Cys	AGG Arg	CAA Gln	ACT Thr 350	TAT Tyr	ATT 1le	10	56
65	GGA Gly	CAG Gln	TAT Tyr 355	AAA Lys	TAC Tyr	TTC Phe	Lys	CTT Leu 360	TCA Ser	AAC Asn	TTG Leu	TTA Leu	AAT Asn 365	GAT Asp	TCT Ser	ATT Ile	11	.04
70	TAT Tyr	AAT Asn	ATA Ile	TCA Ser	GAA Glu	GGC Gly	TAT Tyr	AAT Asn	ATA Ile	AAT Asn	AAT Asn	TTA Leu	AAG Lys	GTA Val	AAT Asn	TTT Phe	11	.52

		370					375					380					
5	AGA Arg 385	GGA Gly	CAG Gln	AAT Asn	GCA Ala	AAT Asn 390	TTA Leu	AAT Asn	CCT Pro	AGA Arg	ATT Ile 395	ATT Ile	ACA Thr	CCA Pro	ATT Ile	ACA Thr 400	1200
10	GGT Gly	AGA Arg	GGA Gly	CTA Leu	GTA Val 405	AAA Lys	AAA Lys	ATC Ile	ATT Ile	AGA Arg 410	TTT Phe	TGT Cys	AAA Lys	AAT Asn	ATT Ile 415	GTT Val	1248
••	TCT Ser	GTA Val	AAA Lys	GGC Gly 420	ATA	AGG Arg	AAA Lys	TCA Ser	ATA Ile 425	TG T Cys	ATC Ile	GAA Glu	ATA Ile	AAT Asn 430	AAT Asn	GGT Gly	1296
15	GAG Glu	TTA Leu	TTT Phe 435	TTT Phe	GTG Val	GCT Ala	TCC Ser	GAG Glu 440	AAT Asn	AGT Ser	TAT Tyr	AAT Asn	GAT Asp 445	GAT Asp	AAT Asn	ATA Ile	1344
20	AAT Asn	ACT Thr 450	CCT Pro	AAA Lys	GAA Glu	ATT Ile	GAC Asp 455	GAT Asp	ACA Thr	GTA Val	ACT Thr	TCA Ser 460	AAT Asn	AAT Asn	AAT Asn	TAT Tyr	1392
25	GAA Glu 465	AAT Asn	GAT Asp	TTA Leu	GAT Asp	CAG Gln 470	GTT Val	ATT Ile	TTA Leu	AAT Asn	TTT Phe 475	AAT Asn	AGT Ser	GAA Glu	TCA Ser	GCA Ala 480	1440
30	CCT Pro	GGA Gly	CTT Leu	TCA Ser	GAT Asp 485	GAA Glu	AAA Lys	TTA Leu	AAT Asn	TTA Leu 490	ACT Thr	ATC Ile	CAA Gln	AAT Asn	GAT Asp 495	GCT Ala	1488
	TAT Tyr	ATA Ile	CCA Pro	AAA Lys 500	TAT Tyr	GAT Asp	TCT Ser	AAT Asn	GGA Gly 505	ACA Thr	AGT Ser	GAT Asp	ATA Ile	GAA Glu 510	CAA Gln	CAT His	1536
35	GAT Asp	GTT Val	AAT Asn 515	GAA Glu	CTT Leu	AAT Asn	GTA Val	TTT Phe 520	TTC Phe	TAT Tyr	TTA Leu	GAT Asp	GCA Ala 525	CAG Gln	AAA Lys	GTG Val	1584
40	CCC Pro	GAA Glu 530	GGT Gly	GAA Glu	AAT Asn	AAT Asn	GTC Val 535	AAT Asn	CTC Leu	ACC Thr	TCT Ser	TCA Ser 540	ATT Ile	GAT Asp	ACA Thr	GCA Ala	1632
45	TTA Leu 545	TTA Leu	GAA Glu	CAA Gln	CCT Pro	AAA Lys 550	ATA Ile	TAT Tyr	ACA Thr	TTT Phe	TTT Phe 555	TCA Ser	TCA Ser	GAA Glu	TTT Phe	ATT Ile 560	1680
50	Asn	Asn	Val	Asn	AAA Lys 565	Pro	Val	Gln	Ala	Ala 570	Leu	Phe	Val	Ser	Trp 575	Ile	1728
	CAA Gln	CAA Gln	GTA Val	TTA Leu 580	GTA Val	GAT Asp	TTT Phe	ACT Thr	ACT Thr 585	GAA Glu	GCT Ala	AAC Asn	CAA Gln	AAA Lys 590	AGT Ser	ACT Thr	1776
55	Val	Asp	Lys 595	Ile	GCA Ala	Asp	Ile	Ser 600	Ile	Val	Val	Pro	Tyr 605	Ile	Gly	Leu	1824
60	Ala	Leu 610	Asn	Ile	GGA Gly	Asn	Glu 615	Ala	Gln	Lys	Gly	Asn 620	Phe	Lys	Asp	Ala	1872
65	Leu 625	Glu	Leu	Leu	GGA Gly	Ala 630	Gly	Ile	Leu	Leu	Glu 635	Phe	Glu	Pro	Glu	Leu 640	1920
70	TTA Leu	ATT Ile	CCT Pro	Thr	ATT Ile 645	TTA Leu	GTA Val	TTC Phe	ACG Thr	ATA Ile 650	AAA Lys	TCT Ser	TTT Phe	TTA Leu	GGT Gly 655	TCA Ser	1968

- 309 -

																	'
	TC1 Sex	GAT Asp	AA7 Asr	AAA Lys 660	, wan	Lys	GTI Val	ATT	AAA Lys 665	Ala	ATA	AAT Asn	AAT Asn	GCA Ala	Leu	AAA Lys	2016
5	GAA Glu	AGA Arg	GAT Asp 675	, 010	AAA Lys	TGG Trp	AAA Lys	GAA Glu 680	vai	TAT Tyr	AGT Ser	TTT Phe	ATA Ile 685	Val	TCG Ser	AAT Asn	2064
10	TGG Trp	Met 690		AAA Lys	ATT	AAT Asn	ACA Thr 695	GIN	TTT Phe	AAT Asn	AAA Lys	AGA Arg 700	Lys	GAA Glu	CAA Gln	ATG Met	2112
15	TAT Tyr 705	CAA Gln	GCT Ala	TTA Leu	CAA Gln	AAT Asn 710	CAA Gln	GTA Val	AAT Asn	GCA Ala	CTT Leu 715	AAA Lys	GCA Ala	ATA Ile	ATA Ile	GAA Glu 720	2160
20		_,0	.,.	ASII	725	TAT Tyr	Int	Leu	GIU	730	Lys	Asn	Glu	Leu	Thr 735	Asn	2208
2.5	-,0	-,-	дор	740	Gru	CAA Gln	116	GIU	745	Glu	Leu	Asn	Gln	Lys 750	Val	Ser	2256
25			755	ASII	ASII	ATA Ile	Asp	760	Pne	Leu	Thr	Glu	Ser 765	Ser	Ile	Ser	2304
30	TAT Tyr	TTA Leu 770	ATG Met	AAA Lys	TTA Leu	ATA Ile	AAT Asn 775	GAA Glu	GTA Val	AAA Lys	ATT Ile	AAT Asn 780	AAA Lys	TTA Leu	AGA Arg	GAA Glu	2352
35	TAT Tyr 785	GAT Asp	GAA Glu	TAA neA	G T T Val	AAA Lys 790	ACG Thr	TAT Tyr	TTA Leu	TTA Leu	GAT Asp 795	TAT Tyr	ATT Ile	ATA Ile	AAA Lys	CAT His 800	2400
40	027	501	116	beu	805	GAG Glu	ser	Gin	Gin	810	Leu	Asn	Ser	Met	Val 815	Ile	2448
	,,,,,	••••	Deu	820	ASII	AGT Ser	11e	Pro	825	Lys	Leu	Ser	Ser	Tyr 830	Thr	Asp	2496
45	GAT Asp	AAA Lys	ATT Ile 835	TTA Leu	ATT 1le	TCA Ser	TAT Tyr	TTT Phe 840	AAT Asn	AAG Lys	TTC Phe	TTT Phe	AAG Lys 845	AGA Arg	ATT Ile	AAA Lys	2544
50	501	850	261	vai	Leu		855	Arg	Tyr	Lys	Asn	Asp 860	Lys	Tyr	Val	Asp	2592
55	865	501	Gry	TYL	ASP	TCA Ser 870	ASN	11e	Asn	He	Asn 875	Gly	Asp	Val	Tyr	Lys 880	2640
60	TAT Tyr	CCA Pro	ACT Thr	AAT Asn	AAA Lys 885	AAT Asn	CAA Gln	TTT Phe	GGA Gly	ATA Ile 890	TAT Tyr	AAT Asn	GAT . Asp	Lys	CTT Leu 895	AGT Ser	2688
	GAA Glu	GTT Val	AAT Asn	ATA Ile 900	TCT Ser	CAA Gln	AAT Asn	GAT Asp	TAC Tyr 905	ATT . Ile	ATA Ile	TAT Tyr	Asp .	AAT Asn 910	AAA Lys	TAT Tyr	2736
65	AAA Lys	AAT Asn	TTT Phe 915	AGT Ser	ATT Ile	AGT Ser	Pne	TGG Trp 920	GTA Val	AGA Arg	ATT Ile	Pro	AAC Asn 925	TAT Tyr	GAT Asp	AAT Asn	2784
70	AAG Lys	ATA Ile	GTA Val	AAT Asn	GTT Val	AAT Asn	AAT Asn	GAA Glu	TAC Tyr	ACT Thr	ATA Ile	ATA Ile	AAT (Asn (TGT Cys	ATG Met	AGG Arg	2832

GAT AAT AAT TCA GGA TGG AAA GTA TCT CTT AAT CAT AAT GAA ATA ATT ASP Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile TGG ACA TTG CAA GAT AAT TCA GGA ATT AAT CAA AAA TTA GCA TTT AAC Trp Thr Leu Gln Asp Asn Ser Gly Ile Asn Gln Lys Leu Ala Phe Asn TAT GGT AAC GCA AAT GGT ATT TCT GAT TAT ATA AAT AAG TGG ATT TTT Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Lle Phe GTA ACT ATA ACT AAT GAT AGA TTA GGA GAT TCT AAA CTT TAT ATT AAT Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn GGA AAT TTA ATA GAT AAA AAA TCA ATT TTA AAT TTA GGT AAT ATT CAT Gly Asn Leu Ile Asp Lys Lys Ser Ile Leu Asn Leu Gly Asn Ile His GTT AGT GAC AAT ATA TTA TTT AAA ATA GTT AAT TGT AGT TAT ACA AGA Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg TAT ATT GGT ATT AGA TAT TTT AAT ATT TTT GAT AAA GAA TTA GAT GAA Tyr lle Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu ACA GAA ATT CAA ACT TTA TAT AAC AAT GAA CCT AAT GCA AAT ATT TTA Thr Glu Ile Gln Thr Leu Tyr Asn Asn Glu Pro Asn Ala Asn Ile Leu AAG GAT TTT TGG GGA AAT TAT TTG CTT TAT GAC AAA GAA TAC TAT TTA Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu TTA AAT GTG TTA AAA CCA AAT AAC TTT ATT AAT AGG AGA ACA GAT TCT Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asn Arg Arg Thr Asp Ser ACT TTA AGC ATT AAT AAT ATA AGA AGC ACT ATT CTT TTA GCT AAT AGA Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg TTA TAT AGT GGA ATA AAA GTT AAA ATA CAA AGA GTT AAT AAT AGT AGT Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser ACT AAC GAT AAT CTT GTT AGA AAG AAT GAT CAG GTA TAT ATT AAT TTT Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe GTA GCC AGC AAA ACT CAC TTA CTT CCA TTA TAT GCT GAT ACA GCT ACC Val Ala Ser Lys Thr His Leu Leu Pro Leu Tyr Ala Asp Thr Ala Thr ACA AAT AAA GAG AAA ACA ATA AAA ATA TCA TCA TCT GGC AAT AGA TTT Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe AAT CAA GTA GTA ATG AAT TCA GTA GGA TGT ACA ATG AAT TTT AAA

Asn Gln Val Val Met Asn Ser Val Gly Cys Thr Met Asn Phe Lys

,	AAT Asn	AAT Asn	AAT AST	GGA Gly	AAT Asn 120	ASI	ATT Ile	GGG Gly	TTG Leu	TTA Lev 121	Gly	TTC Phe	AAG Lys	G GCF Ala	GAT Asp 121	ACT Thr	3648
5	7.7	, ,	ALG	122	0	пр	lyr	ryr	122	1 H18	Met	Arg	Asp	123	Thr	AAC Asn	3696
10	AGC Ser	AAT Asn	GGA Gly 123	FILE	TTT Phe	TGG Trp	AAC Asn	TTT Phe 124	тте	TCT Ser	GAA Glu	GAA Glu	CAT His	Gly	TGG	CAA Gln	3744
15		AAA Lys 125															3753
	(2)	INF	ORMA	TION	FOR	SEQ	ID I	NO : 5	0:								
20			(i) :	(B) LE:	NGTH PE: a	RACTI : 12! amino GY: :	50 ai	mino id	: aci	ds						
25		(ii) I	MOLE	CULE	TYPI	E: p	rote	in								
		(:	xi) s	SEQUI	ENCE	DES	CRIP	rion	: SE	Q ID	NO:	50:					
30	-			Ile	3					10					15		
	Thr	Ile	Leu	Tyr 20	Ile	Lys	Pro	Gly	Gly 25	Cys	Gln	Gln	Phe	Tyr 30	Lys	Ser	
35	Phe	Asn	Ile 35	Met	Lys	Asn	Ile	Trp	Ile	Ile	Pro	Glu	Arg 45	Asn	Val	Ile	
	Gly	Thr 50	Ile	Pro	Gln	Asp	Phe 55	Leu	Pro	Pro	Thr	Ser 60	Leu	Lys	Asn	Gly	
10	Asp 65	Ser	Ser	Tyr	Tyr	Asp 70	Pro	Asn	Tyr	Leu	Gln 75	Ser	Asp	Gln	Glu	Lys 80	
15	Asp	Lys	Phe	Leu	Lys 85	Ile	Val	Thr	Lys	Ile 90	Phe	Asn	Arg	Ile	Asn 95	Asp	
	Asn	Leu	Ser	Gly 100	Arg	Ile	Leu	Leu	Glu 105	Glu	Leu	Ser	Lys	Ala 110	Asn	Pro	
50	Tyr	Leu	Gly 115	Asn	Asp	Asn	Thr	Pro 120	Asp	Gly	Asp	Phe	Ile 125	Ile	Asn	Asp	
	Ala	Ser 130	Ala	Val	Pro	Ile	Gln 135	Phe	Ser	Asn	Gly	Ser 140	Gln	Ser	Ile	Leu	
55	Leu 145	Pro	Asn	Val	Iìe	Ile 150	Met	Gly	Ala	Glu	Pro 155	Asp	Leu	Phe	Glu	Thr 160	
o()	Asn	Ser	Ser	Asn	Ile 165	Ser	Leu	Arg	Asn	Asn 170	Tyr	Met	Pro	Ser	Asn 175	His	
,,,	Gly	Phe	Gly	Ser 180	Ile	Ala	Ile	Val	Thr 185	Phe	Ser	Pro	Glu	Tyr 190	Ser	Phe	
5	Arg	Phe	Lys 195	Asp	Asn	Ser	Met	Asn 200	Glu	Phe	Ile	Gln	Asp 205	Pro	Ala	Leu	
	Thr	Leu 210	Met	His	Glu	Leu	Ile 215	His	Ser	Leu	His	Gly 220	Leu	Tyr	Gly	Ala	
' 0	Lys	Gly	Ile	Thr	Thr	Lys	Tyr	Thr	Ile	Thr	Gln	Lys	Gln	Asn	Pro	Leu	

	225	5				230)				235	,				240	
5	116	∃ Th:	r Asr	ılle	245	Gly	Thi	. Asn	Ile	e Glu 250	ı Glu	Phe	e Lev	Thi	Phe 255	Gly	
	Gly	Th	r Asp	260	Asn	Ile	Ile	Thr	Ser 265	Ala	Gln	Ser	Asn	Asp 270	o Ile	Tyr	
10	Thr	Ası	1 Leu 275	Leu	Ala	Asp	Туг	Lys 280	Lys	Ile	Ala	Ser	Lys 285		ı Ser	Lys	
1.5		290	,				295)				300	1			Glu	
15	505	•				310					315					Asn 320	
20			l Lys		325					330					335		
			Leu	340					345					350			
25			355					360					365				
30		370					375					380					
./0	505		Gln			390					395					400	
35			Gly		405					410					415		
			Lys	420					425					430			
4()			Phe 435					440					445				
45		430	Pro				455					460					
1	403		Asp			4 / 0					475					480	
50			Leu		485					490					495		
			Pro	500					505					510			
55			Asn 515 Gly					520					525				
5()		330	Glu				535					540					
	343					550					555					560	
5			Val		202					570					575		
				380					585					590			
70			Lys 595		a	y	*1C	500	+16	val	val	PLO	Tyr 605	тте	GIY	Leu	

	Ala	610	Asn	Ile	Gly	Asn	Glu 615	Ala	Gln	Lys	Gly	Asn 620	Phe	Lys	Asp	Ala
5	Leu 625	Glu	Leu	Leu	Gly	Ala 630	Gly	Ile	Leu	Leu	Glu 635	Phe	Glu	Pro	Glu	Leu 640
	Leu	Ile	Pro	Thr	Ile 645	Leu	Val	Phe	Thr	Ile 650	Lys	Ser	Phe	Leu	Gly 655	
10	Ser	Asp	Asn	Lys 660	Asn	Lys	Val	Ile	Lys 665	Ala	Ile	Asn	Asn	Ala 670	Leu	Lys
15	Glu	Arg	Asp 675	Glu	Lys	Trp	Lys	Glu 680	Val	Tyr	Ser	Phe	Ile 685	Val	Ser	Asn
	Trp	Met 690	Thr	Lys	Ile	Asn	Thr 695	Gln	Phe	Asn	Lys	Arg 700	Lys	Glu	Gln	Met
20	Tyr 705	Gln	Ala	Leu	Gln	Asn 710	Gln	Val	Asn	Ala	Leu 715	Lys	Ala	Ile	Ile	Glu 720
	Ser	Lys	Tyr	Asn	Ser 725	Tyr	Thr	Leu	Glu	Glu 730	Lys	Asn	Glu	Leu	Thr 735	Asn
25	Lys	Tyr	Asp	Ile 740	Glu	Gln	Ile	Glu	Asn 745	Glu	Leu	Asn	Gln	Lys 750	Val	Ser
30	Ile	Ala	Met 755	Asn	Asn	Ile	Asp	Arg 760	Phe	Leu	Thr	Glu	Ser 765	Ser	Ile	Ser
	Tyr	Leu 770	Met	Lys	Leu	Ile	Asn 775	Glu	Val	Lys	Ile	Asn 780	Lys	Leu	Arg	Glu
35	Tyr 785	Asp	Glu	Asn	Val	Lys 790	Thr	Tyr	Leu	Leu	Asp 795	Tyr	Ile	Ile	Lys	His 800
	Gly	Ser	Ile	Leu	Gly 805	Glu	Ser	Gln	Gln	Glu 810	Leu	Asn	Ser	Met	Val 815	Ile
4()	Asp	Thr	Leu	Asn 820	Asn	Ser	Ile	Pro	Phe 825	Lys	Leu	Ser	Ser	Tyr 830	Thr	Asp
45	Asp	Lys	Ile 835	Leu	Ile	Ser	Tyr	Phe 840	naA	Lys	Phe	Phe	Lys 845	Arg	Ile	Lys
	Ser	Ser 850	Ser	Val	Leu	Asn	Met 855	Arg	Tyr	Lys	Asn	Asp 860	Lys	Tyr	Val	Asp
50	Thr 865	Ser	Gly	Tyr	Asp	Ser 870	Asn	Ile	Asn	Ile	Asn 875	Gly	Asp	Val	Tyr	Lys 880
	туr	Pro	Thr	Asn	Lys 885	Asn	Gln	Phe	Gly	Ile 890	Tyr	Asn	Asp	Lys	Leu 895	Ser
55	Glu	Val	Asn	Ile 900	Ser	Gln	Asn	Asp	Tyr 905	Ile	Ile	Tyr	Asp	Asn 910	Lys	Tyr
60	Lys	Asn	Phe 915	Ser	Ile	Ser	Phe	Trp 920	Val	Arg	Ile	Pro	Asn 925	Tyr	Asp	Asn
	Lys	Ile 930	Val	Asn	Val	Asn	Asn 935	Glu	Tyr	Thr	Ile	Ile 940	Asn	Cys	Met	Arg
65	Λsp 945	Asn	Asn	Ser	Gly	Trp 950	Lys	Val	Ser	Leu	Asn 955	His	Asn	Glu	Ile	11e 960
	Trp	Thr	Leu	Gln	Asp 965	Asn	Ser	Gly	Ile	Asn 970	Gln	Lys	Leu	Ala	Phe 975	Asn
70	Tyr	Gly	Asn	Ala	Asn	Gly	Ile	Ser	Asp	Tyr	Ile	Asn	Lys	Trp	Ile	Phe

				980					985					990			
5	Val	Thr	Ile 995	Thr	Asn	Asp	Arg	Leu 100	Gly 0	Asp	Ser	Lys	Leu 100	Tyr 5	Ile	Asn	
5	Gly	Asn 101	Leu 0	Ile	Asp	Lys	Lys 101	Ser 5	Ile	Leu	Asn	Leu 102		Asn	Ile	His	
10	Val 102	Ser 5	Asp	Asn	Ile	Leu 1030	Phe	Lys	Ile	Val	Asn 103		Ser	Tyr	Thr	Arg 1040	
	Tyr	Ile	Gly	Ile	Arg 104	Tyr 5	Phe	Asn	Ile	Phe 1050	Asp O	Lys	Glu	Leu	Asp 105		
15				1060	J			Asn	1069	5				107	0		
20			107	5				Leu 1080)				1089	5			
		1090	J				1095					1100)				
25	110	5				1110	}	Arg			1115	•				1120	
30					1125	•		Lys		1130)				1135	5	
30				1140)			Lys	1145					1150)		
35			1155	•				Leu 1160					1165				
		11/0	,				1175					1180					
40	1185	•				1190		Ser			1195					1200	
45					1205			Gly		1210					1215		
1				1220					1225					1230			
50	Glu		1235	Pne	Pne	Trp .	Asn	Phe 1240	Ile	Ser	Glu		His 1245		Trp	Gln	
		1250			FOR												
55	(2)		SEQ	UENC	E CH	ARAC'	TERI	0:51 STIC	S:								
50			(B) TY	PE : RAND	nuclo EDNE:	eic SS:	ase j acid doub ar		S							
				ECUL:		PE: 1	DNA	(gen	omic:)							
55		. = 2.7	(A) NAI	ME/K			756									
70								N: S1									
. 5	ATG	CCA	HAA ,	ATT	TAP	AGT T	rtt i	TAA	rat 1	TAP	GAT (CT (GTT A	TAP	GAT A	AGA	48

	Met 1	Pro	Lys	Ile	Asn 5	Ser	Phe	Asn	Tyr	Asn 10	Asp	Pro	Val	Asn	Asp 15	Arg	
5	ACA Thr	ATT	TTA Leu	TAT Tyr 20	116	AAA Lys	CCA Pro	GGC Gly	GGT Gly 25	Cys	CAA Gln	GAA Glu	TTT Phe	TAT Tyr 30	Lys	TCA Ser	96
10	• • • •	7311	35	Met	rys	ASN	ııe	40	Ile	Ile	Pro	Glu	Arg 45	Asn	Val	ATT Ile	144
15	01,	50	1111	PIO	GIII	Asp	55	HIS	Pro	Pro	Thr	Ser 60	Leu	Lys	Asn	GGA Gly	192
	GAT Asp 65	AGT Ser	AGT Ser	TAT Tyr	TAT Tyr	GAC Asp 70	CCT Pro	AAT Asn	TAT Tyr	TTA Leu	CAA Gln 75	AGT Ser	GAT Asp	GAA Glu	GAA Glu	AAG Lys 80	240
20	пор	Arg	rne	Leu	85 85	ATA Ile	vaı	Thr	Lys	90	Phe	Asn	Arg	Ile	Asn 95	Asn	288
25		Dea	Jer	100	GIŸ	ATT Ile	Leu	ren	105	Glu	Leu	Ser	Lys	Ala 110	Asn	Pro	336
30	• : •	De G	115	ASII	Asp	AAT Asn	Thr	120	Asp	Asn	Gln	Phe	His 125	Ile	Gly	Asp	384
35		130	AIA	vai	Giu	ATT Ile	135	Pne	Ser	Asn	Gly	Ser 140	Gln	Asp	Ile	Leu	432
	TTA Leu 145	Pro Pro	AAT Asn	GTT Val	ATT Ile	ATA Ile 150	ATG Met	GGA Gly	GCA Ala	GAG Glu	CCT Pro 155	GAT Asp	TTA Leu	TTT Phe	GAA Glu	ACT Thr 160	480
40	AAC Asn	AGT Ser	TCC Ser	AAT Asn	ATT Ile 165	TCT Ser	CTA Leu	AGA Arg	AAT Asn	AAT Asn 170	TAT Tyr	ATG Met	CCA Pro	AGC Ser	AAT Asn 175	CAC His	528
45	GGT Gly	TTT Phe	GGA Gly	TCA Ser 180	ATA Ile	GCT Ala	ATA Ile	GTA Val	ACA Thr 185	TTC Phe	TCA Ser	CCT Pro	GAA Glu	TAT Tyr 190	TCT Ser	TTT Phe	576
50	AGA Arg	TTT Phe	AAT Asn 195	GAT Asp	TAA nsA	AGT Ser	ATG Met	AAT Asn 200	GAA Glu	TTT Phe	ATT Ile	CAA Gln	GAT Asp 205	CCT Pro	GCT Ala	CTT Leu	624
55	* * * * *	TTA Leu 210	ATG Met	CAT His	GAA Glu	TTA Leu	ATA Ile 215	CAT His	TCA Ser	TTA Leu	CAT His	GGA Gly 220	CTA Leu	TAT Tyr	GGG Gly	GCT Ala	672
	AAA Lys 225	GGG Gly	ATT Ile	ACT Thr	ACA Thr	AAG Lys 230	TAT Tyr	ACT Thr	ATA Ile	ACA Thr	CAA Gln 235	AAA Lys	CAA Gln	AAT Asn	CCC Pro	CTA Leu 240	720
60	ATA Ile	ACA Thr	AAT Asn	ATA Ile	AGA Arg 245	GGT Gly	ACA Thr	AAT Asn	ATT Ile	GAA Glu 250	GAA Glu	TTC Phe	TTA Leu	ACT Thr	TTT Phe 255	GGA Gly	768
65	GGT Gly	ACT Thr	GAT Asp	TTA Leu 260	AAC Asn	ATT Ile	ATT Ile	ACT Thr	AGT Ser 265	GCT Ala	CAG Gln	TCC Ser	AAT Asn	GAT Asp 270	ATC Ile	TAT Tyr	816
70	ACT Thr	ASII	CTT Leu 275	CTA Leu	GCT Ala	GAT Asp	TAT Tyr	AAA Lys 280	AAA Lys	ATA Ile	GCG Ala	TCT Ser	AAA Lys 285	CTT Leu	AGC Ser	AAA Lys	864

	GT: Va	A CA 1 G1 29	u va	A TC	T AA: r Asi	r CCA n Pro	CTA Leu 295	ı Lei	T AA' 1 Asi	T CC	T TA	T AA r Ly 30	s As	T GT p Va	T TI l Ph	T GAA e Glu	912
5	GCI Ala 309	L Ly.	G TA'	T GG/ r Gly	A TT/ / Lei	A GAT Asp 310	rha	A GAT	r GCT o Ala	T AGG	G GG F Gl	y Il	T TA e Ty	T TC r Se	G GT r Va	A AAT 1 Asn 320	
10	••	, ,,,,	ı by:	o Pile	325	Asp	тте	Pne	: Буз	330	E Let	т Ту:	r Se	r Ph	e Th 33	_	
15			J Let	340)	. шуѕ	Phe	GIR	345	Lys	Cys	S Arg	g Gli	1 Th:	r Ty	T ATT	1056
20	4. 7	J. .	355	. Dys	ıyı	Pile	Lys	360	ser	Asn	Le.	ı Leı	369	ı Ası) Se	r ATT	1104
25	.,.	370)	Jei	Giu	GIY	375	Asn	116	Asn	Asn	380	Lys	Va]	Ası	T TTT 1 Phe	1152
25	385	Oly		ASII	AIA	390	Leu	Asn	Pro	Arg	11e 395	Ile	Thr	Pro) Ile	400	1200
30	u.,	arg	Gly	Leu	405	ьys	Lys	11e	ile	Arg 410	Phe	Cys	Lys	Asn	11e	•	1248
35	,	vai		420	116	AGG Arg	Lys	ser	425	Cys	Ile	Glu	Ile	Asn 430	Asn	Gly	1296
40	0.4	Deu	435	Pile	val	GCT Ala	ser	440	Asn	Ser	Tyr	Asn	Asp 445	Asp	Asn	Ile	1344
	71011	450	FIO	пуъ	GIU		455	Asp	Thr	Val	Thr	Ser 460	Asn	Asn	Asn	Tyr	1392
45	465	ASII	изр	neu	Asp	CAG Gln 470	vaı	IIe	Leu	Asn	Phe 475	Asn	Ser	Glu	Ser	Ala 480	1440
50		O. y	Dea	361	485	GAA . Glu	Lys	Leu	Asn	Leu 490	Thr	Ile	Gln	Asn	Asp 495	Λla	1488
55	.,.	110	FIO	500	TYI	GAT Asp	ser	Asn	505	Thr	Ser	Asp	Ile	Glu 510	Gln	His	1536
60		, u <u> </u>	515	GIU	Leu	AAT (Asn '	vaı	520	Pne	Tyr	Leu	Asp	Ala 525	Gln	Lys	Val	1584
	CCC Pro	GAA Glu 530	G GT Gly	GAA Glu	AAT Asn	AAT (Asn \	GTC A Val A	AAT Asn	CTC Leu	ACC Thr	TCT Ser	TCA Ser 540	ATT Ile	GAT Asp	ACA Thr	GCA Ala	1632
65	545	Dea	GIU	GIN	PIO .	AAA A Lys 1 550	ile :	ryr	Thr	Phe	Phe 555	Ser	Ser	Glu	Phe	Ile 560	1680
70	AAT . Asn .	AAT Asn	GTC Val	AAT Asn	AAA Lys	CCT (Pro \	GTG (/al (CAA (Gln .	GCA (GCA Ala	TTA Leu	TTT Phe	GTA Val	AGC Ser	TGG Trp	ATA Ile	1728

	•				565					570					575		
5	CAA Gln	CAA Gln	GTG Val	TTA Leu 580	GTA Val	GAT Asp	TTT Phe	ACT Thr	ACT Thr 585	GAA Glu	GCT Ala	AAC Asn	CAA Gln	AAA Lys 590	AGT Ser	ACT Thr	1776
10	GTT Val	GAT Asp	AAA Lys 595	ATT Ile	GCA Ala	GAT Asp	ATT Ile	TCT Ser 600	ATA Ile	GTT Val	GTT Val	CCA Pro	TAT Tyr 605	ATA Ile	GGT Gly	CTT Leu	1824
	GCT Ala	TTA Leu 610	AAT Asn	ATA Ile	GGA Gly	AAT Asn	GAA Glu 615	GCA Ala	CAA Gln	AAA Lys	GGA Gly	AAT Asn 620	TTT Phe	AAA Lys	GAT Asp	GCA Ala	1872
15	CTT Leu 625	GAA Glu	TTA Leu	TTA Leu	GGA Gly	GCA Ala 630	GGT Gly	ATT Ile	TTA Leu	TTA Leu	GAA Glu 635	TTT Phe	GAA Glu	CCC Pro	GAG Glu	CTT Leu 640	1920
20	TTA Leu	ATT Ile	CCT Pro	ACA Thr	ATT Ile 645	TTA Leu	GTA Val	TTC Phe	ACG Thr	ATA Ile 650	AAA Lys	TCT Ser	TTT Phe	TTA Leu	GGT Gly 655	TCA Ser	1968
25	TCT Ser	GAT Asp	AAT Asn	AAA Lys 660	TAA neA	AAA Lys	GTT Val	ATT Ile	AAA Lys 665	GCA Ala	ATA Ile	AAT Asn	AAT Asn	GCA Ala 670	TTG Leu	AAA Lys	2016
30	GAA Glu	AGA Arg	GAT Asp 675	GAA Glu	AAA Lys	TGG Trp	AAA Lys	GAA Glu 680	GTA Val	TAT Tyr	AGT Ser	TTT Phe	ATA Ile 685	GTA Val	TCG Ser	AAT Asn	2064
. -	Trp	ме t 690	Thr	Lys	Ile	Asn	Thr 695	Gln	Phe	Asn	Lys	Arg 700	AAA Lys	Glu	Gln	Met	2112
35	705	GIn	Ala	Leu	Gln	710	Gln	Val	Asn	Ala	Ile 715	Lys	ACA Thr	Ile	Ile	Glu 720	2160
40	TCT Ser	AAG Lys	TAT Tyr	AAT Asn	AGT Ser 725	TAT Tyr	ACT Thr	TTA Leu	GAG Glu	GAA Glu 730	AAA Lys	AAT Asn	GAG Glu	CTT Leu	ACA Thr 735	AAT Asn	2208
45	Lys	Tyr	Asp	11e 740	Lys	Gln	Ile	Glu	Asn 745	Glu	Leu	Asn	CAA Gln	Lys 750	Val	Ser	2256
50	ile	Ala	Met 755	Asn	Asn	Ile	Asp	Arg 760	Phe	Leu	Thr	Glu	AGT Ser 765	Ser	Ile	Ser	2304
	lyr	770	Met	Lys	Leu	Ile	775	Glu	Val	Lys	Ile	Asn 780	AAA Lys	Leu	Arg	Glu	2352
55	785	Asp	Glu	Asn	Val	Lys 790	Thr	Tyr	Leu	Leu	Asn 795	Tyr	ATT Ile	Ile	Gln	His 800	2400
60	GIY	ser	11e	Leu	61y 805	Glu	Ser	Gln	Gln	Glu 810	Leu	Asn	TCT Ser	Met	Val 815	Thr	2448
65	Asp	Thr	Leu	Asn 820	Asn	Ser	Ile	Pro	Phe 825	Lys	Leu	Ser	TCT Ser	Tyr 830	Thr	Asp	2496
70	GAT Asp	AAA Lys	ATT Ile 835	TTA Leu	ATT	TCA Ser	TAT Tyr	TTT Phe 840	AAT Asn	AAA Lys	TTC Phe	TTT Phe	AAG Lys 845	AGA Arg	ATT Ile	AAA Lys	2544

- 318 -

•	AGT Ser	AGT Ser 850	Ser	GTT Val	TTA Leu	AAT Asn	ATG Met 855	AGA Arg	TAT	AAA Lys	AAT Asn	GAT Asp 860	AAA Lys	TAC	GTA Val	GAT Asp	2592
5	ACT Thr 865	Ser	GGA Gly	TAT	GAT Asp	TCA Ser 870	AAT Asn	ATA Ile	AAT Asn	ATT	AAT Asn 875	Gly	GAT Asp	GTA Val	TAT	AAA Lys 880	2640
10	TAT Tyr	CCA Pro	ACT Thr	AAT Asn	AAA Lys 885	AAT Asn	CAA Gln	TTT Phe	GGA Gly	ATA Ile 890	TAT	AAT Asn	GAT Asp	AAA Lys	CTT Leu 895	AGT Ser	2688
15	G AA Glu	GTT Val	AAT Asn	ATA Ile 900	Ser	CAA Glņ	AAT Asn	GAT Asp	TAC Tyr 905	ATT Ile	ATA Ile	TAT Tyr	GAT Asp	AAT Asn 910	AAA Lys	TAT Tyr	2736
20	AAA Lys	AAT Asn	TTT Phe 915	AGT Ser	ATT Ile	AGT Ser	TTT Phe	TGG Trp 920	GTA Val	AGA Arg	ATT Ile	CCT Pro	AAC Asn 925	TAT Tyr	GAT Asp	AAT Asn	2784
	AAG Lys	ATA Ile 930	GTA Val	AAT Asn	GTT Val	AAT Asn	AAT Asn 935	GAA Glu	TAC Tyr	ACT Thr	ATA Ile	ATA Ile 940	AAT Asn	TGT Cys	ATG Met	AGA Arg	2832
[°] 25	GAT Asp 945	AAT Asn	AAT Asn	TCA Ser	GGA Gly	TGG Trp 950	AAA Lys	GTA Val	TCT Ser	CTT Leu	AAT Asn 955	CAT His	AAT Asn	GAA Glu	ATA Ile	ATT Ile 960	2880
30	TGG Trp	ACA Thr	TTG Leu	CAA Gln	GAT Asp 965	AAT Asn	GCA Ala	GGA Gly	ATT Ile	AAT Asn 970	CAA Gln	AAA Lys	TTA Leu	GCA Ala	TTT Phe 975	AAC Asn	. 2928
35	TAT Tyr	GGT Gly	AAC Asn	GCA Ala 980	AAT Asn	GGT Gly	ATT Ile	TCT Ser	GAT Asp 985	TAT Tyr	ATA Ile	AAT Asn	AAG Lys	TGG Trp 990	ATT Ile	TTT Phe	2976
40	GTA Val	ACT Thr	ΛTA Ile 995	ACT Thr	AAT Asn	GAT Asp	AGA Arg	TTA Leu 1000	Gly	GAT Asp	TCT Ser	AAA Lys	CTT Leu 1005	Tyr	ATT Ile	AAT Asn	3024
	GGA Gly	AAT Asn 1010	Leu	ATA Ile	GAT Asp	CAA Gln	AAA Lys 1015	Ser	ATT Ile	TTA Leu	AAT Asn	TTA Leu 1020	Gly	AAT Asn	ATT Ile	CAT His	3072
45	GTT Val 1025	Ser	GAC Asp	AAT Asn	ATA Ile	TTA Leu 1030	Phe	AAA Lys	ATA Ile	GTT Val	AAT Asn 1035	Cys	AGT Ser	TAT Tyr	ACA Thr	AGA Arg 1040	3120
50	TAT Tyr	ATT Ile	GG T Gly	ATT Ile	AGA Arg 1045	TAT Tyr	TTT Phe	AAT Asn	ATT Ile	Phe	Asp	AAA Lys	Glu	Leu	Asp	Glu	3168
55	ACA Thr	GAA Glu	ATT Ile	CAA Gln 1060	Thr	TTA Leu	TAT Tyr	AGC Ser	AAT Asn 1065	Glu	CCT Pro	AAT Asn	ACA Thr	AAT Asn 1070	Ile	TTG Leu	3216
60	AAG Lys	GAT Asp	TTT Phe 1075	Trp	GGA Gly	AAT Asn	TAT Tyr	TTG Leu 1080	Leu	TAT Tyr	GAC Asp	Lys	GAA Glu 1085	Tyr	TAT Tyr	TTA Leu	3264
	TTA Leu	AAT Asn 1090	Val	TTA Leu	AAA Lys	Pro	AAT Asn 1095	Asn	TTT Phe	ATT Ile	GAT Asp	AGG Arg 1100	AGA Arg	AAA Lys	GAT Asp	TCT Ser	3312
65	ACT Thr 1105	Leu	AGC Ser	ATT Ile	AAT Asn	AAT Asn 1110	Ile	AGA Arg	AGC Ser	ACT Thr	ATT Ile 1115	Leu	TTA Leu	GCT Ala	AAT Asn	AGA Arg 1120	3360
70	TTA Leu	TAT Tyr	AGT Ser	GGA Gly	ATA Ile	AAA Lys	GTT Val	AAA Lys	ATA Ile	CAA Gln	AGA Arg	GTT Val	AAT Asn	AAT Asn	AGT Ser	AGT Ser	3408

	112	5	1130	1135
5	ACT AAC GAT AAT CTT Thr Asn Asp Asn Leu 1140	GTT AGA AAG AA Val Arg Lys As 11	n Asp Gin val Tyr	· ·
10	GTA GCC AGC AAA ACT Val Ala Ser Lys Thr 1155	CAC TTA TTT CC. His Leu Phe Pro 1160	A TTA TAT GCT GAT O Leu Tyr Ala Asp 116	Thr Ala Thr
	ACA AAT AAA GAG AAA Thr Asn Lys Glu Lys 1170	ACA ATA AAA ATA Thr Ile Lys Ilo 1175	A TCA TCA TCT GGC e Ser Ser Ser Gly 1180	AAT AGA TTT 3552 Asn Arg Phe
15	AAT CAA GTA GTA GTT Asn Gln Val Val 1185	ATG AAT TCA GTA Met Asn Ser Val 1190	A GGA AAT AAT TGT L Gly Asn Asn Cys 1195	ACA ATG AAT 3600 Thr Met Asn 1200
20	TTT AAA AAT AAT AAT Phe Lys Asn Asn Asn 120	OTA WOIL WRIT TIE	GGG TTG TTA GGT Gly Leu Leu Gly 1210	TTC AAG GCA 3648 Phe Lys Ala 1215
25	GAT ACT GTA GTT GCT Asp Thr Val Val Ala 1220	AGT ACT TGG TAT Ser Thr Trp Tyr 122	Tyr Thr His Met	AGA GAT CAT 3696 Arg Asp His 1230
30	ACA AAC AGC AAT GGA Thr Asn Ser Asn Gly 1235	TGT TTT TGG AAC Cys Phe Trp Asn 1240	TTT ATT TCT GAA Phe Ile Ser Glu 1245	Glu His Gly
	TGG CAA GAA AAA TAA Trp Gln Glu Lys 1250			3759
35	(2) INFORMATION FOR	SEQ ID NO:52:		
40	(A) LEN (B) TYF (D) TOF	CHARACTERISTICS GTH: 1252 amino E: amino acid OLOGY: linear	: acids	
15	(ii) MOLECULE (xi) SEQUENCE	TYPE: protein DESCRIPTION: SE	O ID NO.52.	
45	Met Pro Lys Ile Asn			
50	Thr Ile Leu Tyr Ile	Lys Pro Gly Gly 25	Cys Gln Glu Phe	15 Tyr Lys Ser
	Phe Asn Ile Met Lys			30 Asn Val Ile
55	Gly Thr Thr Pro Gln 50		Pro Thr Ser Leu	Lys Asn Gly
60	Asp Ser Ser Tyr Tyr 65	Asp Pro Asn Tyr 70		Glu Glu Lys 80
00	Asp Arg Phe Leu Lys 85	Ile Val Thr Lys	Ile Phe Asn Arg	
65	Asn Leu Ser Gly Gly 100	Ile Leu Leu Glu 105		
	Tyr Leu Gly Asn Asp . 115	Asn Thr Pro Asp 120		
70	Ala Ser Ala Val Glu	Ile Lys Phe Ser	Asn Gly Ser Gln	Asp Ile Leu

	-	130					135					140				
5	Leu 145	Pro	Asn	Val	Ile	Ile 150	Met	Gly	Ala	Glu	Pro 155	Asp	Leu	Phe	Glu	Th:
	Asn	Ser	Ser	Asn	Ile 165	Ser	Leu	Arg	Asn	Asn 170	туr	Met	Pro	Ser	Asn 175	Hi
10	Gly	Phe	Gly	Ser 180	Ile	Ala	Ile	Val	Thr 185	Phe	Ser	Pro	Glu	Tyr 190	Ser	Pho
	Arg	Phe	Asn 195	Asp	Asn	Ser	Met	Asn 200	Glu	Phe	Ile	Gln	Asp 205	Pro	Ala	Lev
15	Thr	Leu 210	Met	His	Glu	Leu	Ile 215	His	Ser	Leu	His	Gly 220	Leu	Tyr	Gly	Ala
20	Lys 225	Gly	Ile	Thr	Thr	Lys 230	Tyr	Thr	Ile	Thr	Gln 235	Lys	Gln	Asn	Pro	Let 240
	Ile	Thr	Asn	Ile	Arg 245	Gly	Thr	Asn	Ile	Glu 250	Glu	Phe	Leu	Thr	Phe 255	Gly
25	Gly	Thr	Asp	Leu 260	Asn	Ile	Ile	Thr	Ser 265	Ala	Gln	Ser	Asn	Asp 270	Ile	Тут
• •			275			Asp _.		280					285			
30		290				Pro	295					300				
35	305					Asp 310					315					320
-					325	Asp				330					335	
40				340		Lys			345					350		
1.5			355			Phe		360					365			
45		370				Gly	375					380				
50	385					Asn 390					395					400
					405	Lys				410					415	
55				420		Arg			425					430		
60			435			Ala		440					445			
		450				Ile	455					460				
65	465					Gln 470					475					480
					485	Glu				490					495	
7()	iyr	116	Pro	Lys 500	Tyr	Asp	ser	Asn	Gly 505	Thr	Ser	Asp	Ile	Glu 510	Gln	His

	Ası	va.	l As: 51:	n Glu 5	u Lei	ı Ası	n Va	1 Phe 520	Phe	е Туг	Leu	Asp	Ala 525	Glr	ı Ly:	s Val
5	Pro	530	u Gly	y Glu	ı Asn	a Asr	1 Va. 53	l Ası 5	ı Lei	1 Thr	Ser	Ser 540	Ile	. Asp	Th	Ala
	Leu 545	Leu S	ı Glu	ı Glr	Pro	550	s Ile	Э Туг	Thr	. Phe	Phe 555	Ser	Ser	Glu	Phe	lle 560
10										5/0					579	
15									202	,				590		Thr
								800					605			Leu
20							015					620				Ala
25.											635					Leu 640
23.					Ile 645					650					655	
30					Asn				003					670		
					Lys			000					685			
35					Ile		0,5					700				
40					Gln	, 10					/15					720
•••					Ser 725					730					735	
45				. 10	Lys				/45					750		
•					Asn			760					765			
50.					Leu		, , ,					780				
55					Val						/95					800
					Gly 805					810					815	
60				020	Asn				825					830		
					Ile			840				1	845			
65					Leu .		000				1	860				
70	Thr 865					6 / 0					875					880
70	Tyr	Pro	Thr	Asn	Lys	Asn	Gln	Phe	Gly	Ile '	Tyr 1	Asn A	Asp i	Lys :	Leu	Ser

					885					890)				895	5
5	Glu	Val	Asn	11e 900	Ser	Gln	Asn	Asp	Tyr 905	: Ile	lle	Tyr	Asp	Asn 910		Tyr
	Lys	Asn	915	Ser	Ile	Ser	Phe	Trp 920	Val	Arg	Ile	Pro	925		Asp	Asn
10	Lys	Ile 930	Val	Asn	Val	Asn	Asn 935	Glu	Tyr	Thr	Ile	Ile 940	Asn	Cys	Met	Arg
	Asp 945	Asn	Asn	Ser	Gly	Trp 950	Lys	Val	Ser	Leu	Asn 955	His	Asn	Glu	Ile	Ile 960
15					965					970					975	
20				960					985					990		Phe
			223					1000	U				100	5		Asn
25		101	U	Ile			101	5				102	0			
20	102.	,				1030	,				103	5				Arg 1040
30				Ile	1045	•				105	0				105	5
35			,	Gln 106	נ				106	5				1070)	
			107					1080)				1085	5		
40	Leu	1090	J				1095	,				1100)			
45	1105	'		Ile		1110)				1115	5				1120
40	Leu				1125					1130)				1135	5
50	Thr			1140)				1145	5				1150)	
	Val		1155	•				1160					1165			
55	Thr	11/0	,				11/5					1180)			
60	Asn 1185					1190					1195					1200
00	Phe				1205					1210					1215	
65	Asp			1220					1225	1				1230		
	Thr		1235		Gly	Cys	Phe	Trp . 1240	Asn	Phe	Ile		Glu 1245	Glu	His	Gly
70	Trp	G1n 1250		rys												

	(2)) IN	FORM	ATIO	N FO	R SE	Q ID	NO:	53:								
5		(:		(A) 1 (B) 1 (C) 5	LENG: TYPE STRAI	CHARI TH: 1 : nuc NDEDI LOGY:	1463 Cleic NESS:	base ac: do:	e pa: id	irs							
10		(ii) MC	LECU (A) [JLE T DESCE	YPE:	oth	ner m	nucle sc =	eic a "DNA	acid 4"						
15			(B) L	IAME/	KEY: ION:	108	314		ID N	10 : 5 3	:					
30																TAACAA	60
20	TTC	CCCT	CTA	GAAA	TAAT	тт т	GTTT	'AACT	T TA	LAGAA	GGAG	ATA	TACC		Gly	CAT His	116
25.	CAT His	CAT His		CAT	CAT His	CAT His	CAT His	HIS	CAC His	AGC Ser	AGC Ser	GGC Gly 15	CAT His	ATC Ile	GAA Glu	GGT Gly	164
30	20				561	25	AIG	reu	ser	ser	Tyr 30	Thr	Asp	Asp	Lys	ATT Ile 35	212
35			-	- 7 -	40	ASIL	Буѕ	Pne	Pne	Lys 45	Arg	Ile	Lys	Ser	Ser 50		260
	Val	TTA Leu	AAT Asn	ATG Met 55	AGA Arg	TAT Tyr	AAA Lys	AAT Asn	GAT Asp 60	Lys	TAC Tyr	GTA Val	GAT Asp	ACT Thr 65	TCA Ser	G GA Gly	308
40	TAT Tyr	GAT Asp	TCA Ser 70	AAT Asn	ATA Ile	AAT Asn	ATT Ile	AAT Asn 75	GGA Gly	GAT Asp	GTA Val	TAT Tyr	AAA Lys 80	TAT Tyr	CCA Pro	ACT Thr	356
45	TAA Asn	AAA Lys 85	AAT Asn	CAA Gln	TTT Phe	GGA Gly	ATA Ile 90	TAT Tyr	AAT Asn	GAT Asp	AAA Lys	CTT Leu 95	AGT Ser	GAA Glu	GTT Val	AAT Asn	404
50	ATA Ile 100	TCT Ser	CAA Gln	AAT Asn	GAT Asp	TAC Tyr 105	ATT Ile	ATA Ile	TAT Tyr	GAT Asp	AAT Asn 110	AAA Lys	TAT Tyr	AAA Lys	AAT Asn	TTT Phe 115	452
55	AGT Ser	ATT Ile	AGT Ser	TTT Phe	TGG Trp 120	GTA Val	AGA Arg	ATT Ile	CCT Pro	AAC Asn 125	TAT Tyr	GAT Asp	AAT Asn	AAG Lys	ATA Ile 130	GTA Val	500
	AAT Asn	GTT Val	AAT Asn	AAT Asn 135	GAA Glu	TAC Tyr	ACT Thr	ATA Ile	ATA Ile 140	AAT Asn	TGT Cys	ATG Met	AGG Arg	GAT Asp 145	AAT Asn	AAT Asn	548
60	TCA Ser	GGA Gly	TGG Trp 150	AAA Lys	GTA Val	TCT Ser	CTT Leu	λΑΤ Asn 155	CAT His	AAT Asn	GAA Glu	ATA Ile	ATT Ile 160	TCC	ACA Thr	TTG Leu	596
65	CAA Gln	GAT Asp 165	AAT Asn	TCA Ser	GGA Gly	ATT Ile	AAT Asn 170	CAA Gln	AAA Lys	TTA Leu	GCA Ala	TTT Phe 175	AAC Asn	TAT Tyr	GGT Gly	AAC Asn	644
70	GCA Ala 180	TAA Asn	GGT Gly	ATT Ile	TC T Ser	GAT Asp 185	TAT Tyr	ATA lle	AAT Asn	AAG Lys	TGG Trp 190	ATT Ile	TTT Phe	GTA Val	ACT Thr	ATA Ile 195	692

,	ACT Thr	AAT Asn	Asp	AGA Arg	Leu 200	GGA Gly	GAT Asp	TCT Ser	Lys	Leu 205	TAT	ATT	TAA neA	GGA Gly	AAT Asn 210	TTA Leu	740
5	ATA Ile	GAT Asp	AAA Lys	AAA Lys 215	TCA Ser	ATT Ile	TTA Leu	AAT Asn	TTA Leu 220	GGT Gly	AAT Asn	ATT Ile	CAT His	GTT Val	AGT Ser	GAC Asp	788
10	AAT Asn	ATA Ile	TTA Leu 230	TTT Phe	AAA Lys	ATA Ile	GTT Val	AAT Asn 235	TG T Cys	AGT Ser	TAT Tyr	ACA Thr	AGA Arg 240	TAT Tyr	ATT Ile	GGT Gly	836
15	ATT Ile	AGA Arg 245	TAT Tyr	TTT Phe	AAT Asn	ATT Ile	TTT Phe 250	GAT Asp	AAA Lys	GAA Glu	TTA Leu	GAT Asp 255	GAA Glu	ACA Thr	GAA Glu	ATT Ile	884
20	CAA Gln 260	ACT Thr	TTA Leu	TAT Tyr	AAC Asn	AAT Asn 265	GAA Glu	CCT Pro	AAT Asn	GCA Ala	AAT Asn 270	ATT Ile	TTA Leu	AAG Lys	GAT Asp	TTT Phe 275	932
	TGG Trp	GGA Gly	AAT Asn	TAT Tyr	TTG Leu 280	CTT Leu	TAT Tyr	GAC Asp	AAA Lys	GAA Glu 285	TAC Tyr	TAT Tyr	TTA Leu	TTA Leu	AAT Asn 290	GTG Val	980
25	TTA Leu	AAA Lys	CCA Pro	AAT Asn 295	AAC Asn	TTT Phe	ATT Ile	AAT Asn	AGG Arg 300	AGA Arg	ACA Thr	GAT Asp	TCT Ser	ACT Thr 305	TTA Leu	AGC Ser	1028
30	ATT Ile	AAT Asn	AAT Asn 310	ATA Ile	AGA Arg	AGC Ser	ACT Thr	ATT Ile 315	CTT Leu	TTA Leu	GCT Ala	AAT Asn	AGA Arg 320	TTA Leu	TAT Tyr	AGT Ser	1076
35	GGA Gly	ATA Ile 325	AAA Lys	GTT Val	AAA Lys	ATA Ile	CAA Gln 330	AGA Arg	GTT Val	AAT Asn	AAT Asn	AGT Ser 335	AGT Ser	ACT Thr	AAC Asn	GAT Asp	1124
40	AAT Asn 340	CTT Leu	GTT Val	AGA Arg	AAG Lys	AAT Asn 345	GAT Asp	CAG Gln	GTA Val	TAT Tyr	ATT Ile 350	AAT Asn	TTT Phe	GTA Val	GCC Ala	AGC Ser 355	1172
	AAA Lys	ACT Thr	CAC His	TTA Leu	CTT Leu 360	CCA Pro	TTA Leu	TAT Tyr	GCT Ala	GAT Asp 365	ACA Thr	GCT Ala	ACC Thr	ACA Thr	AAT Asn 370	AAA Lys	1220
45	GAG Glu	AAA Lys	ACA Thr	ATA Ile 375	AAA Lys	ATA Ile	TCA Ser	TCA Ser	TCT Ser 380	GGC Gly	AAT Asn	AGA Arg	TTT Phe	AAT Asn 385	CAA Gln	GTA Val	1268
50	GTA Val	GTT Val	ATG Met 390	AAT Asn	TCA Ser	GTA Val	GGA Gly	AAT Asn 395	TGT Cys	ACA Thr	ATG Met	AAT Asn	TTT Phe 400	AAA Lys	AAT Asn	AAT Asn	1316
55	AAT Asn	GGA Gly 405	AAT Asn	TAA Asn	ATT Ile	GGG Gly	TTG Leu 410	TTA Leu	GGT Gly	TTC Phe	AAG Lys	GCA Ala 415	GAT Asp	ACT Thr	GTA Val	G T T Val	1364
50	GCT Ala 420	AGT Ser	ACT Thr	TGG Trp	TAT Tyr	TAT Tyr 425	ACA Thr	CAT His	ATG Met	AGA Arg	GAT Asp 430	AAT Asn	ACA Thr	AAC Asn	AGC Ser	AAT Asn 435	1412
	GGA Gly	TTT Phe	TTT Phe	TGG Trp	AAC Asn 440	TTT Phe	ATT Ile	TCT Ser	GAA Glu	GAA Glu 445	CAT His	GGA Gly	TGG Trp	CAA Gln	GAA Glu 450	AAA Lys	1460
55	TAA																1463

(2) INFORMATION FOR SEQ ID NO:54:

70

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 451 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear (ii) MOLECULE TYPE: protein 5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:54: Met Gly His His His His His His His His Ser Ser Gly His 10 Ile Glu Gly Arg His Met Ala Ser Met Ala Leu Ser Ser Tyr Thr Asp Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys 15 Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp 20 Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser 25 Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn 30 Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg 35 Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile Trp Thr Leu Gln Asp Asn Ser Gly Ile Asn Gln Lys Leu Ala Phe Asn 4() Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn 45 Gly Asn Leu Ile Asp Lys Lys Ser Ile Leu Asn Leu Gly Asn Ile His 215 50 Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu 55 Thr Glu Ile Gln Thr Leu Tyr Asn Asn Glu Pro Asn Ala Asn Ile Leu 265 Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu 60 280 Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asn Arg Arg Thr Asp Ser 65 Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg 315 Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser

330

70

	Thr	Asn	Asp	Asn 340		Val	Arg	Lys	Asn 345		Gln	Val	Туr	Ile 350		Phe		
5	Val	Ala	Ser 355	Lys	Thr	His	Leu	Leu 360		Leu	Tyr	Ala	Asp 365	Thr	Ala	Thr		
	Thr	Asn 370	Lys	Glu	Lys	Thr	Ile 375	Lys	Ile	Ser	Ser	Ser 380	Gly	Asn	Arg	Phe		
10	Asn 385	Gln	Val	Val	Val	Met 390	Asn	Ser	Val	Gly	Asn 395	Cys	Thr	Met	Asn	Phe 400		
15	Lys	Asn	Asn	Asn	Gly 405	Asn	Asn	Ile	Gly	Leu 410	Leu	Gly	Phe	Lys	Ala 415	Asp		
•.,	Thr	Val	Val	Ala 420	Ser	Thr	Trp	Tyr	Tyr 425	Thr	His	Met	Arg	Asp 430	Asn	Thr		
20	Asn	Ser	Asn 435	Gly	Phe	Phe	Trp	Asn 440	Phe	Ile	Ser	Glu	Glu 445	His	Gly	Trp		
	Gln	Glu 450	Lys															
25	(2)	INF	ORMA?	rion	FOR	SEQ	ID 1	10 : 5 !	5:									
30		(i·	(E	A) LE 3) TY 2) S7	CE CHENGTH YPE: TRANI	H: 14 nucl	172 l leic ESS:	ase acid doub	pai:	rs								
35		(ii)	MOI (7		LE TY													
	•	(ix)		4) NA	E: AME/H DCATI			.146	5 3									
40		(x1)	SEC	QUENC	E DE	ESCRI	TPTIC	ON: 5	SEQ :	D NO	0:55	:						
	AGAT	rctco	GAT (CCCGC	GAAA	AT TA	ATA	GAC	CAC	TAT	AGGG	GAAT	TGTC	SAG (GGAT	raacaa		60
45	TTC	CCT	ATS	TAAAT	ГААТТ	rt to	STTT#	ACT	LAT T	AGAAG	5GAG	ATAT	TACC		GGC Gly		1	.16
50	CAT His	CAT His 5	CAT His	CAT His	CAT His	CAT His	CAT His 10	CAT His	CAC His	AGC Ser	AGC Ser	GGC Gly 15	CAT His	ATC Ile	GAA Glu	GGT Gly	1	.64
55	CGT Arg 20	CAT His	ATG Met	GCT Ala	AGC Ser	ATG Met 25	GCT Ala	CTT Leu	TCT Ser	TCT Ser	TAT Tyr 30	ACA Thr	GAT Asp	GAT Asp	AAA Lys	ATT Ile 35	2	12
	TTA Leu	ATT Ile	TCA Ser	TAT Tyr	TTT Phe 40	AAT Asn	AAA Lys	TTC Phe	TTT Phe	AAG Lys 45	AGA Arg	ATT lle	AAA Lys	AGT Ser	AGT Ser 50	TCA Ser	2	60
60	GTT Val	TTA Leu	AAT Asn	ATG Met 55	AGA Arg	TAT Tyr	AAA Lys	AAT Asn	GAT Asp 60	AAA Lys	TAC Tyr	GTA Val	GAT Asp	ACT Thr 65	TCA Ser	GGA Gly	3	0.6
65	TAT Tyr	GAT Asp	TCA Ser 70	AAT Asn	ATA Ile	AAT Asn	ATT Ile	AAT Asn 75	GGA Gly	GAT Asp	GTA Val	TAT Tyr	AAA Lys 80	TAT Tyr	CCA Pro	ACT Thr	3	5€
70	AAT Asn	AAA Lys 85	AAT Asn	CAA Gln	TTT Phe	GGA Gly	ATA 11e 90	TAT Tyr	AAT Asn	GAT Asp	AAA Lys	CTT Leu 95	AGT Ser	GAA Glu	GTT Val	AAT Asn	4	04

,	ATA 116	A TC	r CAJ	A AAT n Asr	GAT Asp	TAC Tyr	TIE	ATA	A TAT	GA?	AA7 Asr	ı Lys	A TAT	r AAA	A AAT S Asr	TTT	452
5	AG1 Ser	ATT	Γ AG] ⊇ Ser	TTT Phe	TGG Trp	vai	AGA Arg	ATT	CCT Pro	AAC Asr 125	TAT		AA7 Asi	T AAC	ATA	115 GTA Val	500
10				135	010		1111	116	140	ASI	Cys	Met	Arg	Asp 145	Asn	AAT Asn	548
15		•	150	-7-	•••		Deu	155	піѕ	ASN	GIU	lie	11e	Trp	Thr	T T G Leu	596
20		165			GGA Gly	116	170	GIN	гÀг	ren	Ala	Phe 175	Asn	Tyr	Gly	Asn	644
25	180		<i></i> ,		TCT Ser	185	TYL	116	Asn	rys	Trp 190	Ile	Phe	Val	Thr	11e 195	692
25			ПОР	9	TTA Leu 200	GIY	ASP	ser	Lys	205	Tyr	Ile	Asn	Gly	Asn 210	Leu	740
30			0111	215	TCA Ser	116	Leu	ASN	220	GIY	Asn	Ile	His	Val 225	Ser	Asp	788
35			230	riie	AAA Lys	116	vai	235	Cys	Ser	Tyr	Thr	Arg 240	Tyr	Ile	Gly	836
40		245	- 7 -	rne	AAT Asn	116	250	Asp	Lys	Glu	Leu	Asp 255	Glu	Thr	Glu	Ile	884
45	260		Deu	. 7 .	AGC Ser	265	GIU	Pro	Asn	Thr	270	Ile	Leu	Lys	Asp	Phe 275	932
4.1		O.J.	A311	TYL	TTG Leu 280	Leu	TYE	Asp	Lys	285	Tyr	Tyr	Leu	Leu	Asn 290	Val	980
50	Deu	273	710	295	AAC Asn	Pne	116	Asp	300	Arg	Lys	Asp	Ser	Thr 305	Leu	Ser	1028
55			310	116	AGA Arg	sei	inr	315	Leu	Leu	Ala	Asn	Arg 320	Leu	Tyr	Ser	1076
60	,	325	Dy S	vai	AAA Lys	iie ,	330	arg	vaı	Asn	Asn	Ser 335	Ser	Thr	Asn	Asp	1124
65	AAT Asn 340	Leu	vai	Arg	Lys	345	Asp (GIN	Val	туг	11e 350	Asn	Phe	Val	Ala	Ser 355	1172
65	AAA Lys	••••		Leu	360	PIO .	Leu	ryr	Ala	365	Thr	Ala	Thr	Thr	Asn 370	Lys	1220
70	GAG Glu	AAA Lys	ACA Thr	ATA Ile	AAA . Lys	ATA 1	TCA ' Ser :	TCA Ser	TCT Ser	GGC Gly	AAT Asn	AGA Arg	TTT Phe	AAT Asn	CAA Gln	GTA Val	1268

				37	5				380)				38	5		
5	GT/ Va	A GT l Va	T AT 1 Me 39	c As	T TC	A GTA	A GG#	A AA: / Asi 399	n Asr	r TG:	T AC s Th	A ATG	G AA' t Ası 400	r TT		A AAT s Asn	1316
10	AA: Ası	r AA n As 40		A AA' y Asi	T AA: n Asi	TATI Ile	GGC Gly 410	Let	3 TTA 1 Let	A GGT u Gly	r TT y Ph	C AAG e Ly: 41!	s Ala	A'GA' As _l	T AC	T GTA r Val	1364
	GT1 Val 420		T AG' a Se:	r AC	T TGC	TAT Tyr 425	Tyr	Thr	CAT His	ATC Met	AG Arg 430	g Asp	r CAT	ACI Thi	AA A	C AGC n Ser 435	1412
15	AAT Asn	GG/	A TG: / Cys	T TT:	T TGC ⇒ Trp 440	ASD	TTT Phe	ATT	TCT Ser	GAA Glu 445	ı Glı	A CAT	r GGA Gly	TGC Trp	G CA/ O Gl: 450	A GAA n Glu	1460
20	AAA Lys		AAAG(CTT													1472
	(2)	INE			1 FOR												
125			(i)	(A	JENCE () LE () TY () TO	NGTH PE:	: 45 amin	2 am o ac	ino id	: acid	s						
30					CULE					Q ID	NO:	56 :					
35	-									10					15		
	11e	Glu	Gly	Arg 20	His	Met	Ala	Ser	Met 25	Ala	Leu	Ser	Ser	Tyr 30	Thr	Asp	
40			23		Ile			40					45				
	Ser	Ser 50	Ser	Val	Leu	Asn	Met 55	Arg	Tyr	Lys	Asn	Asp 60	Lys	туг	Val	Asp	
45	0,5				Asp	70					75					80	
50	Tyr	Pro	Thr	Asn	Lys 85	Asn	Gln	Phe	Gly	Ile 90	Tyr	Asn	Asp	Lys	Leu 95	Ser	
	Glu	Val	Asn	Ile 100	Ser	Gln	Asn	Asp	Tyr 105	Ile	Ile	туr	Asp	Asn 110	Lys	Tyr	
55	Lys	Asn	Phe 115	Ser	Ile	Ser	Phe	Trp 120	Val	Arg	Ile	Pro	Asn 125	Tyr	Asp	Asn	
	Lys	11e 130	Val	Asn	Val	Asn	Asn 135	Glu	Tyr	Thr	Ile	Ile 140	Asn	Cys	Met	Arg	
60	Asp 145	Asn	Asn	Ser	Gly	Trp 150	Lys	Val	Ser	Leu	Asn 155	His	Asn	Glu	Ile	Ile 160	
65	Trp	Thr	Leu	Gln	Asp 165	Asn	Λla	Gly	Ile	Asn 170	Gln	Lys	Leu	Λla	Phe 175	Asn	
	Tyr	Gly	Asn	Ala 180	Asn	Gly	Ile	Ser	Asp 185	Tyr	Ile	Asn		Trp 190	Ile	Phe	
70	Val	Thr	Ile 195	Thr	Asn	Asp	Arg	Leu 200	Gly	Asp	Ser	Lys	Leu 205	Tyr	Ile	Asn	

	Gly	Asn 210	Leu	Ile	Asp	Gln	Lys 215	Ser	Ile	Leu	Asn	Leu 220	Gly	Asn	Ile	His		
5	Val 225	Ser	Asp	Asn	Ile	Leu 230	Phe	Lys	Ile	Val	Asn 235	Cys	Ser	Tyr	Thr	Arg 240	•	
	Туг	Ile	Gly	Ile	Arg 245	Tyr	Phe	Asn	Ile	Phe 250	Asp	Lys	Glu	Leu	Asp 255	Glu		
10	Thr	Glu	Ile	Gln 260	Thr	Leu	Tyr	Ser	Asn 265	Glu	Pro	Asn	Thr	Asn 270	Ile	Leu		
15	Lys	Asp	Phe 275	Trp	Gly	Asn	Tyr	Leu 280	Leu	Tyr	Asp	Lys	Glu 285	Tyr	Tyr	Leu		
	Leu	Asn 290	Val	Leu	Lys	Pro	Asn 295	Asn	Phe	Ile	Asp	Arg 300	Arg	Lys	Asp	Ser		
20	Thr 305	Leu	Ser	Ile	Asn	Asn 310	Ile	Arg	Ser	Thr	Ile 315	Leu	Leu	Ala	Asn	Arg 320		
	Leu	Tyr	Ser	Gly	Ile 325	Lys	Val	Lys	Ile	Gln 330	Arg	Val	Asn	Asn	Ser 335	Ser		
25	Thr	Asn	Asp	Asn 340	Leu	Val	Arg	Lys	Asn 345	Asp	Gln	Val	Tyr	Ile 350	Asn	Phe		
30	Val	Ala	Ser 355	Lys	Thr	His	Leu	Phe 360	Pro	Leu	Tyr	Ala	Asp 365	Thr	Ala	Thr		
	Thr	Asn 370	Lys	Glu	Lys	Thr	11e 375	Lys	Ile	Ser	Ser	Ser 380	Gly	Asn	Arg	Phe		
35	Asn 385	Gln	Val	Val	Val	Met 390	Asn	Ser	Val	Gly	Asn 395	Asn	Cys	Thr	Met	Asn 400		
	Phe ·	Lys	Asn	Asn	Asn 405	Gly	Asn	Asn	Ile	Gly 410	Leu	Leu	Gly	Phe	Lys 415	Ala		
40	Asp	Thr	Val	Val 420	Ala	Ser	Thr	Trp	Tyr 425	Tyr	Thr	Hıs	Met	Arg 430	Asp	Hıs		
45	Thr	Asn	Ser 435	Asn	Gly	Cys	Phe	Trp 440	Asn	Phe	Ile	Ser	Glu 445	Glu	His	Gly		
	Trp	Gln 450	Glu	Lys														
50	(2)		RMAT															
55		(1)	(E	L) LE L) TY L) ST L) TO	NGTH PE: RAND	: 31 nucl EDNE	bas eic SS:	e pa acid sino	irs									
		(ii)	MOL		E TY	PE:	othe	r nu	clei	c ac	id							
60		(xi)	SEC	UENC	E DE	SCRI	PTIC	N· S	EO T	ם אכ	57.							
	CGCC		CT C															7.1
			RMAT															31
65			SEC (A	UENC	E CH NGTH	ARAC I: 29 nucl	TERI bas	STIC e pa	S: irs									
70			(C) SI	POLO	EDNE GY:	SS: line	sing ar	jle									

- 330 -

70

	•	(i:	i) M	OLECT	ULE :	TYPE	ot)	ner m	nucle	eic a "DN#	cid							
5		(x)	i) SI	EQUE	NCE I	DESC	RIPTI	ON:	SEQ	ID N	1 0 : 58	3:						
٠,	GCA	AGCI	TTT	ATT	rttci	rtg (CATO	CATO	3									29
	(2)	INF	ORM	OITA	N FOR	SEC) ID	NO : 5	9:					1				-
10		(i	1	(A) I (B) T	ENGT	CHARA TH: 3 nuc	876 :leic	base aci	pai d	.rs								
15		(ii				OGY : YPE :		_	nomi	.c)								
20			:) FE	ATUR	E: IAME/	KEY:	CDS											
		(xi) SE	QUEN	CE D	ESCR	IPTI	ON:	SEQ	ID N	0:59	:						
'25	ATG Met 1	CCA Pro	ATA	ACA Thr	ATT Ile	ASD	AAC Asn	TTT Phe	AAT Asn	TAT Tyr 10	TCA Ser	GAT Asp	CCT Pro	GTT Val	GAT Asp 15	AAT Asn		48
30	AAA Lys	TAA Asn	ATT	TTA Leu 20	Tyr	TTA Leu	GAT Asp	ACT Thr	CAT His 25	Leu	TAA naA	ACA Thr	CTA Leu	GCT Ala 30	Asn	GAG Glu		96
35	CCT Pro	GAA Glu	AAA Lys 35	Ala	TTT Phe	CGC Arg	ATT Ile	ACA Thr 40	GGA Gly	AAT Asn	ATA Ile	TGG Trp	GTA Val 45	ATA Ile	CCT Pro	GAT Asp	•	144
	AGA Arg	TTT Phe 50	TCA Ser	AGA Arg	AAT Asn	TCT Ser	AAT Asn 55	CCA Pro	AAT Asn	TTA Leu	AAT Asn	AAA Lys 60	CCT Pro	CCT Pro	CGA Arg	GTT Val		192
40	ACA Thr 65	AGC Ser	CCT Pro	AAA Lys	AGT Ser	GGT Gly 70	TAT Tyr	TAT Tyr	GAT Asp	CCT Pro	AAT Asn 75	TAT Tyr	TTG Leu	AGT Ser	ACT Thr	GAT Asp 80		240
45	TCT Ser	GAC Asp	AAA Lys	GAT Asp	ACA Thr 85	TTT Phe	TTA Leu	AAA Lys	GAA Glu	ATT Ile 90	ATA Ile	AA G Lys	TTA Leu	TTT Phe	AAA Lys 95	AGA Arg		288
50	ATT Ile	AAT Asn	TCT Ser	AGA Arg 100	GAA Glu	ATA Ile	GGA Gly	GAA Glu	GAA Glu 105	TTA Leu	ATA Ile	TAT Tyr	AGA Arg	CTT Leu 110	TCG Ser	ACA Thr		336
55	GAT Asp	ATA Ile	CCC Pro 115	TTT Phe	CCT Pro	GGG Gly	AAT Asn	AAC Asn 120	AAT Asn	ACT Thr	CCA Pro	ATT Ile	AAT Asn 125	ACT Thr	TTT Phe	GAT Asp		384
<i>J J</i>	TTT Phe	GAT Asp 130	GTA Val	GAT Asp	TTT Phe	AAC Asn	AGT Ser 135	GTT Val	GAT Asp	GTT Val	AAA Lys	ACT Thr 140	AGA Arg	CAA Gln	GGT Gly	A AC Asn		432
60	AAC Asn 145	TGG Trp	GTT Val	AAA Lys	ACT Thr	GGT Gly 150	AGC Ser	ATA Ile	AAT Asn	CCT Pro	AGT Ser 155	GTT Val	ATA Ile	ATA Ile	ACT Thr	GGA Gly 160		480
65	CCT Pro	AGA Arg	GAA Glu	AAC Asn	ATT Ile 165	ATA Ile	GAT Asp	CCA Pro	GAA Glu	ACT Thr 170	TCT Ser	ACG Thr	TTT Phe	AAA Lys	TTA Leu 175	N COTT		528

	AAC	TAA : Asn	ACT Thr	TTT Phe 180	MId	GCA Ala	CAA Gln	GAA Glu	GGA Gly 185	Phe	GGT Gly	GCT Ala	TTA Leu	TCA Ser 190	Ile	ATT	576
5	TCA Ser	ATA Ile	TCA Ser 195	FIO	AGA Arg	TTT Phe	ATG Met	CTA Leu 200	Thr	TAT Tyr	AGT Ser	`AAT Asn	GCA Ala 205	Thr	' AAT Asn	GAT Asp	624
10	GTA Val	GGA Gly 210	GIU	GGT Gly	AGA Arg	TTT Phe	TCT Ser 215	AAG Lys	TCT Ser	GAA Glu	TTT Phe	TGC Cys 220	ATG Met	GAT Asp	CCA Pro	ATA Ile	672
15	CTA Leu 225		TTA Leu	ATG Met	CAT His	GAA Glu 230	CTT Leu	AAT Asn	CAT His	GCA Ala	ATG Met 235	His	AAT Asn	TTA Leu	TAT	GGA Gly 240	720
20			110	110	245	wab	GIN	Inr	116	250	Ser	GTA Val	Thr	Ser	Asn 255	Ile	768
		- , -	561	260	TYL	ASII	vai	rys	265	Glu	Tyr	GCA Ala	Glu	11e 270	Tyr	Ala	816
25		J.,	275	FIO	1111	116	ASP	280	11e	Pro	Lys	AGT Ser	Ala 285	Arg	Lys	Tyr	864
30	1	290	Giu	Lys	Ala	rea	295	Tyr	Tyr	Arg	Ser	ATA Ile 300	Ala	Lys	Arg	Leu	912
35	305	Jei	116		inr	310	Asn	Pro	Ser	Ser	Phe 315	AAT Asn	Lys	Tyr	Ile	Gly 320	960
40	Giu	Tyl	цуѕ	GIN	325	Leu	11e	Arg	Lys	Tyr 330	Arg	TTC Phe	Val	Val	Glu 335	Ser	1008
15	361	GIY	GIU	340	Inr	vai	Asn	Arg	45 345	Lys	Phe	GTT Val	Glu	Leu 350	Tyr	Asn	1056
45	014	Deu	355	GIII	116	Pue	inr	360	Pne	Asn	Tyr	GCT Ala	Lys 365	Ile	Tyr	Asn	1104
50	vai	370	ASII	Arg	Lys	IIe	375	Leu	Ser	Asn	Val	TAT Tyr 380	Thr	Pro	Val	Thr	1152
55	385	ASII	rre	Leu	Asp	390	Asn	Val	Tyr	Asp	11e 395	CAA Gln	Asn	Gly	Phe	Asn 400	1200
60	116	PIO	цуѕ	ser	405	Leu	Asn	Val	Leu	Phe 410	Met	GGT Gly	Gln	Asn	Leu 415	Ser	1248
45	n. g	ASII	PIO	420	Leu	Arg	Lys	Val	Λsn 425	Pro	Glu	AAT Asn	Met	Leu 430	Tyr	Leu	1296
65	7116	1111	435	PHE	Cys	HIS	ràs	440	IIe	Asp	Gly	AGA Arg	Ser 445	Leu	Tyr	Asn	1344
70	AAA Lys	ACA Thr	TTA Leu	GAT Asp	TGT Cys	AGA Arg	GAG Glu	CTT Leu	TTA Leu	GTT Val	AAA Lys	AAT Asn	ACT Thr	GAC Asp	TTA Leu	CCC Pro	1392

450 455 460 TTT ATA GGT GAT ATT AGT GAT GTT AAA ACT GAT ATA TTT TTA AGA AAA 1440 Phe Ile Gly Asp Ile Ser Asp Val Lys Thr Asp Ile Phe Leu Arg Lys 470 475 GAT ATT AAT GAA GAA ACT GAA GTT ATA TAC TAT CCG GAC AAT GTT TCA 1488 Asp Ile Asn Glu Clu Thr Glu Val Ile Tyr Tyr Pro Asp Asn Val Ser 10 GTA GAT CAA GTT ATT CTC AGT AAG AAT ACC TCA GAA CAT GGA CAA CTA Val Asp Gln Val Ile Leu Ser Lys Asn Thr Ser Glu His Gly Gln Leu 1536 15 GAT TTA TTA TAC CCT AGT ATT GAC AGT GAG AGT GAA ATA TTA CCA GGG 1584 Asp Leu Leu Tyr Pro Ser Ile Asp Ser Glu Ser Glu Ile Leu Pro Gly 520 GAG AAT CAA GTC TTT TAT GAT AAT AGA ACT CAA AAT GTT GAT TAT TTG 1632 20 Glu Asn Gln Val Phe Tyr Asp Asn Arg Thr Gln Asn Val Asp Tyr Leu AAT TCT TAT TAT TAC CTA GAA TCT CAA AAA CTA AGT GAT AAT GTT GAA 1680 Asn Ser Tyr Tyr Tyr Leu Glu Ser Gln Lys Leu Ser Asp Asn Val Glu 25 GAT TTT ACT TTT ACG AGA TCA ATT GAG GAG GCT TTG GAT AAT AGT GCA 1728 Asp Phe Thr Phe Thr Arg Ser Ile Glu Glu Ala Leu Asp Asn Ser Ala 565 570 30 AAA GTA TAT ACT TAC TTT CCT ACA CTA GCT AAT AAA GTA AAT GCG GGT Lys Val Tyr Thr Tyr Phe Pro Thr Leu Ala Asn Lys Val Asn Ala Gly 1776 580 35 GTT CAA GGT GGT TTA TTT TTA ATG TGG GCA AAT GAT GTA GTT GAA GAT 1824 Val Gln Gly Gly Leu Phe Leu Met Trp Ala Asn Asp Val Val Glu Asp 600 TTT ACT ACA AAT ATT CTA AGA AAA GAT ACA TTA GAT AAA ATA TCA GAT 1872 40 Phe Thr Thr Asn Ile Leu Arg Lys Asp Thr Leu Asp Lys Ile Ser Asp GTA TCA GCT ATT ATT CCC TAT ATA GGA CCC GCA TTA AAT ATA AGT AAT 1920 Val Ser Ala Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Ser Asn 45 625 TCT GTA AGA AGA GGA AAT TTT ACT GAA GCA TTT GCA GTT ACT GGT GTA 1968 Ser Val Arg Arg Gly Asn Phe Thr Glu Ala Phe Ala Val Thr Gly Val 50 ACT ATT TTA TTA GAA GCA TTT CCT GAA TTT ACA ATA CCT GCA CTT GGT 2016 Thr Ile Leu Leu Glu Ala Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly 665 GCA TTT GTG ATT TAT AGT AAG GTT CAA GAA AGA AAC GAG ATT ATT AAA 2064 Ala Phe Val Ile Tyr Ser Lys Val Gln Glu Arg Asn Glu Ile Ile Lys 680 ACT ATA GAT AAT TGT TTA GAA CAA AGG ATT AAG AGA TGG AAA GAT TCA 2112 60 Thr Ile Asp Asn Cys Leu Glu Gln Arg Ile Lys Arg Trp Lys Asp Ser 700 695 TAT GAA TGG ATG ATG GGA ACG TGG TTA TCC AGG ATT ATT ACT CAA TTT 2160 Tyr Glu Trp Met Met Gly Thr Trp Leu Ser Arg Ile Ile Thr Gln Phe 65 710 AAT AAT ATA AGT TAT CAA ATG TAT GAT TCT TTA AAT TAT CAG GCA GGT Asn Asn Ile Ser Tyr Gln Met Tyr Asp Ser Leu Asn Tyr Gln Ala Gly 2208

70

	GCA Ala	A ATO	AAA Lys	A GC1 8 Ala 740	,-	A ATA	GAI Asp	TTA Leu	GAF Glu 745	ı ıyı	T AAA	A AAI S Lys	A TAT	TCA Ser	Gly	A AGT / Ser	2256
	GAT Asp	AAA Lys	GAA Glu 755		ATA	AAA Lys	AGT Ser	CAA Gln 760	val	GAA Glu	AAT Asn	TTA Let	A AAA Lys 765	Asn	AG1	TTA Leu	2304
10	GAT Asp	GTA Val 770	AAA Lys	ATT Ile	TCG Ser	GAA Glu	GCA Ala 775	ATG Met	AAT Asn	' AAT Asn	ATA Ille	AAT Asn 780	ı Lys	TTT Phe	ATA Ile	CGA Arg	2352
15	GAA Glu 785	TGT Cys	TCC Ser	GTA Val	ACA Thr	TAT Tyr 790	reu	TTT Phe	AAA Lys	AAT Asn	ATG Met 795	Leu	CCT Pro	AAA Lys	GTA Val	ATT Ile 800	2400
20			200	A311	805	File	АБР	Arg	Asn	810	Lys	Ala	Lys	Leu	Ile 815		2448
2.5			· iop	820	1112	WOII	TIE	TTÉ	825	Val	Gly	Glu	Val	Asp 830	Lys	TTA Leu	2496
25.	- 2 -		835	Vai	VOII	ASII	ser	840	GIn	Asn	Thr	Ile	Pro 845	Phe	Asn		2544
30		850	- , -	****	ASII	ASII	855	reu	Leu	Lys	Asp	11e 860	Ile	Asn	Glu		259 2
35	865	- 12		110	ASII	870	ser	Lys	тте	Leu	Ser 875	Leu	CAA Gln	Asn	Arg	Lys 880	2640
40			Jeu	vai	885	1111	ser	GIY	lyr	890	Ala	Glu	GTG Val	Ser	Glu 895	Glu	2688
15	- 4			900	Deu	ASII	PIO	116	905	Pro	Phe	Asp	TTT Phe	Lys 910	Leu	Gly	2736
45		•	915	GIU	Азр	Arg	GIY	920	vai	ile	Val	Thr	CAG Gln 925	Asn	Glu	Asn	2784
50		930	- 7 -	ASII	ser	Mec	935	GIU	Ser	Phe	Ser	11e 940	AGT Ser	Phe	Trp	Ile	2832
55	945		71511	шуз	11.5	950	261	ASN	Leu	Pro	955	Tyr	ACT Thr	Ile	Ile	Asp 960	2880
60		7.	2,5	7311	965	361	GIY	rrp	ser	970	GIA	Ile	ΛΤΤ Ile	Ser	Asn 975	Phe	2928
<i>(</i> =	TTA Leu			980	beu	гуѕ	GIN	ASN	985	Asp	Ser	Glu	Gln	Ser 990	Ile	Asn	2976
65	TTT Phe	OCI	TAT Tyr 995	GAT Asp	ATA Ile	TCA . Ser .	ASI .	AAT Asn 1000	Ala	CCT Pro	GGA Gly	TAC Tyr	AAT Asn 1005	AAA Lys	TGG Trp	TTT Phe	3024
70	TTT Phe	GTA Val	ACT Thr	GTT Val	ACT Thr	AAC . Asn .	AAT . Asn	ATG Met	ATG Met	GGA Gly	TAA Asn	ATG Met	AAG Lys	ATT	TAT Tyr	ATA Ile	3072

	1010	1015	1020	
5	1025	1030	GTT AAA GAA CTA ACT GGA ATT Val Lys Glu Leu Thr Gly Ile 1035 1040	3120
10	10	45	ATA AAT AAA ATT CCA GAT ACC Ile Asn Lys Ile Pro Asp Thr 1050 1055	3168
	1060	1065	1070	3216
15	1075	1080	GGT AAA GAT ATT AAT ATA TTA Gly Lys Asp Ile Asn Ile Leu 1085	3264
20	TTT AAT AGC TTG CAM Phe Asn Ser Leu Glr 1090	A TAT ACT AAT GTT (I Tyr Thr Asn Val \ 1095	GTA AAA GAT TAT TGG GGA AAT Val Lys Asp Tyr Trp Gly Asn 1100	3312
125	1105	1110	ATG GTT AAT ATA GAT TAT TTA Met Val Asn Ile Asp Tyr Leu 1115 1120	3360
30	112	5 1	CAA ATT GTT TTT AAT ACA CGT Gln Ile Val Phe Asn Thr Arg 1130	3408
	AGA AAT AAT AAT GAC Arg Asn Asn Asn Asp 1140	TTC AAT GAA GGA T Phe Asn Glu Gly T 1145	TAT AAA ATT ATA ATA AAA AGA Tyr Lys Ile Ile Ile Lys Arg 1150	3456
35	1155	1160	GTA CGA GGA GGA GAT ATT TTA Val Arg Gly Gly Asp Ile Leu 1165	3504
40	1170	1175	CCA TAT AAT TTG TTT ATG AAG la Tyr Asn Leu Phe Met Lys 1180	3552
45	1185	1190	GT ACT GAA GAT ATA TAT GCT er Thr Glu Asp Ile Tyr Ala 1195 1200	3600
50	1205	i iii Lys Asp 1	TA AAT GAT AAT ATT ATA TTT le Asn Asp Asn Ile Ile Phe 210 1215	3648
	1220	1225	AT TAC GCA TCT CAA ATA TTT yr Tyr Ala Ser Gln Ile Phe 1230	3696
55	1235	1240	CT GGA ATA TGT TCA ATA GGT er Gly Ile Cys Ser Ile Gly 1245	3744
60	1250 The Alg	1255	GG TAT AGA CAC AAT TAT TTG rp Tyr Arg His Asn Tyr Leu 1260	3792
65	1265	1270	CT TCA TTA TTA GAA TCA ACA la Ser Leu Leu Glu Ser Thr 1275 1280	3840
70	TCA ACT CAT TGG GGT Ser Thr His Trp Gly 1285	Pne Val Pro Val Se	ET GAA TAA Er Glu 290	3876

(2) INFORMATION FOR SEQ ID NO:60:

5			(i)	(B) LE) TY	NGTH PE :	RACT : 12 amin GY:	91 a o ac	mino id	: aci	ds					
10				MOLE												
10		. (:	xi)	SEQU	ENCE	DES	CRIP	TION	: SE	Q ID	NO:	60:				
	Met 1	Pro	Ile	Thr	Ile 5	Asn	Asn	Phe	Asn	Tyr 10	Ser	Asp	Pro	Val	Asp 15	
15	Lys	Asn	Ile	Leu 20	Tyr	Leu	Asp	Thr	His 25	Leu	Asn	Thr	Leu	Ala 30	Asn	Gli
20	Pro	Glu	Lys 35	Ala	Phe	Arg	Ile	Thr 40	Gly	Asn	Ile	Trp	Val 45	Ile	Pro	Ası
	Arg	Phe 50	Ser	Arg	Asn	Ser	Asn 55	Pro	Asn	Leu	Asn	Lys 60	Pro	Pro	Arg	Va]
25	Thr 65	Ser	Pro	Lys	Ser	Gly 70	Tyr	Tyr	Asp	Pro	Asn 75	Tyr	Leu	Ser	Thr	Ası 80
	Ser	Asp	Lys	Asp	Thr 85	Phe	Leu	Lys	Glu	Ile 90	Ile	Lys	Leu	Phe	Lys 95	Arç
30	Ile	Asn	Ser	Arg 100	Glu	Ile	Gly	Glu	Glu 105	Leu	Ile	туг	Arg	Leu 110	Ser	Thi
35	Asp	Ile	Pro 115	Phe	Pro	Gly	Asn	Asn 120	Asn	Thr	Pro	Ile	Asn 125	Thr	Phe	Asp
	Phe	Asp '130	Val	Asp	Phe	Asn	Ser 135	Val	Asp	Val	Lys	Thr 140	Arg	Gln	Gly	Asr
40	Asn 145	Trp	Val	Lys	Thr	Gly 150	Ser	Ile	Asn	Pro	Ser 155	Val	Ile	Ile	Thr	Gly 160
	Pro	Arg	Glu	Asn	Ile 165	Ile	Asp	Pro	Glu	Thr 170	Ser	Thr	Phe	Lys	Leu 175	Thr
45	Asn	Asn	Thr	Phe 180	Ala	Ala	Gln	Glu	Gly 185	Phe	Gly	Ala	Leu	Ser 190	lie	Ile
50	Ser	lle	Ser 195	Pro	Arg	Phe	Met	Leu 200	Thr	Tyr	Ser	Asn	Ala 205	Thr	Asn	Asp
	Val	Gly 210	Glu	Gly	Arg	Phe	Ser 215	Lys	Ser	Glu	Phe	Cys 220	Met	Asp	Pro	Ile
55	Leu 225	Ile	Leu	Met	His	Glu 230	Leu	Asn	His	Ala	Met 235	His	Asn	Leu	Tyr	Gly 240
•	Ile	Ala	lle	Pro	Asn 245	Asp	Gln	Thr	Ile	Ser 250	Ser	Val	Thr	Ser	Asn 255	Ile
60	Phe	Tyr	Ser	Gln 260	Tyr	Asn	Val	Lys	Leu 265	Glu	Tyr	Ala	Glu	11e 270	Tyr	Ala
65	Phe	Gly	Gly 275	Pro	Thr	Ile	Asp	Leu 280	Ile	Pro	Lys	Ser	Ala 285	Arg	Lys	Tyr
	Phe	Glu 290	Glu	Lys	Ala	Leu	Asp 295	Tyr	Tyr	Arg	Ser	Ile 300	Ala	Lys	Arg	Leu
70	Asn 305	Ser	Ile	Thr	Thr	Ala 310	Asn	Pro	Ser	Ser	Phe 315	Asn	Lys	Tyr	Ile	Gly 320

	Glu	Tyr	Lys	Gln	Lys 325	Leu	Ile	Arg	Lys	Tyr 330	Arg	Phe	Val	Val	Glu 335	
5	Ser	Gly	Glu	Val 340	Thr	Val	Asn	Arg	Asn 345		Phe	Val	Glu	Leu 350		Ası
	Glu	Leu	Thr 355	Gln	Ile	Phe	Thr	Glu 360	Phe	Asn	Tyr	Ala	Lys 365	Ile	Tyr	Ası
10	Val.	Gln 370	Asn	Arg	Lys	Ile	Tyr 375	Leu	Ser	Asn	Val	Tyr 380		Pro	Val	Thi
15	Ala 385	Asn	Ile	Leu	Asp	Asp 390	Asn	Val	Tyr	Asp	Ile 395	Gln	Asn	Gly	Phe	Asr 400
1.7	Ile	Pro	Lys	Ser	Asn 405	Leu	Asn	Val	Leu	Phe 410	Met	Gly	Gln	Asn	Leu 415	Ser
20	Arg	Asn	Pro	Ala 420	Leu	Arg	Lys	Val	Asn 425	Pro	Glu	Asn	Met	Leu 430	Tyr	Leu
	Phe	Thr	Lys 435	Phe	Cys	His	Lys	Ala 440	Ile	Asp	Gly	Arg	Ser 445	Leu	Tyr	Asr
25	Lys	Thr 450	Leu	Asp	Cys	Arg	Glu 455	Leu	Leu	Val	Lys	Asn 460	Thr	Asp	Leu	Pro
30	Phe 465	Ile	Gly	Asp	Ile	Ser 470	Asp	Val	Lys	Thr	Asp 475	Ile	Phe	Leu	Arg	Lys 480
J. (*	Asp	Ile	Asn	Glu	Glu 485	Thr	Glu	Val	Ile	Tyr 490	Tyr	Pro	Asp	Asn	Val 495	Ser
35	Val	Asp	Gln	Val 500	Ile	Leu	Ser	Lys	Asn 505	Thr	Ser	Glu	His	Gly 510	Gln	Leu
	Asp	Leu	Leu 515	Tyr	Pro	Ser	Ile	Asp 520	Ser	Glu	Ser	Glu	Ile 525	Leu	Pro	Gly
1 ()	Glu	Asn 530	Gln	Val	Phe	Tyr	Asp 535	Asn	Arg	Thr	Gln	Asn 540	Val	Asp	Tyr	Leu
1 5	Asn 545	Ser	Tyr	Tyr	Tyr	Leu 550	Glu	Ser	Gln	Lys	Leu 555	Ser	Asp	Asn	Val	Glu 560
7. /	Asp	Phe	Thr	Phe	Thr 565	Arg	Ser	Ile	Glu	Glu 570	Ala	Leu	Asp	Asn	Ser 575	Ala
50	Lys	Val	Tyr	Thr 580	Tyr	Phe	Pro	Thr	Leu 585	Ala	Asn	Lys	Val	Asn 590	Ala	Gly
	Val	Gln	Gly 595	Gly	Leu	Phe	Leu	Met 600	Trp	Ala	Asn	Asp	Val 605	Val	Glu	Asp
55	Phe	Thr 610	Thr	Asn	Ile	Leu	Arg 615	Lys	Asp	Thr	Leu	Asp 620	Lys	Ile	Ser	Asp
50	Val 625	Ser	Ala	Ile	Ile	Pro 630	Tyr	Ile	Gly	Pro	Ala 635	Leu	Asn	Ile	Ser	Asn 640
	Ser	Val	Arg	Arg	Gly 645	Asn	Phe	Thr	Glu	Ala 650	Phe	Ala	Val	Thr	Gly 655	Val
55	Thr	Ile	Leu	Leu 660	Glu	Ala	Phe	Pro	Glu 665	Phe	Thr	Ile	Pro	Ala 670	Leu	Gly
	Ala	Phe	Val 675	Ile	Tyr	Ser	Lys	Val 680	Gln	Glu	Arg	Asn	Glu 685	Ile	Ile	Lys
70	Thr	Ile	qzA	Asn	Cvs	Leu	Glu	Gln	Ara	Tle	Lvs	Ara	Trn	LVS	Δsn	Ser

		690					695	•				700)			
5						710					715	•				Phe 720
•										730				•	735	
10									745					750		Ser
	Asp	Lys	Glu 755	Asn	Ile	Lys	Ser	Gln 760	Val	Glu	Asn	Leu	Lys 765	Asn	Ser	Leu
15	Asp	Val 770	Lys	Ile	Ser	Glu	Ala 775	Met	Asn	Asn	Ile	Asn 780	Lys	Phe	Ile	Arg
20	Glu 785	Cys	Ser	Val	Thr	Tyr 790	Leu	Phe	Lys	Asn	Met 795	Leu	Pro	Lys	Val	Ile 800
	Asp	Glu	Leu	Asn	Glu 805	Phe	Asp	Arg	Asn	Thr 810	Lys	Ala	Lys	Leu	11e 815	Asn
25	Leu	Ile	Asp	Ser 820	His	Asn	Ile	Ile	Leu 825	Val	Gly	Glu	Val	Asp 830	Lys	Leu
	Lys	Ala	Lys 835	Val	Asn	Asn	Ser	Phe 840	Gln	Asn	Thr	Ile	Pro 845	Phe	Asn	Ile
30	Phe	Ser 850	Tyr	Thr	Asn	Asn	Ser 855	Leu	Leu	Lys	Asp	Ile 860	Ile	Asn	Glu	Tyr
35	Phe 865	Asn	Asn	Ile	Asn	Asp 870	Ser	Lys	Ile	Leu	Ser 875	Leu	Gln	Asn	Arg	Lys 880
	Asn	Thr	Leu	Val	Asp 885	Thr	Ser	Glγ	Tyr	Asn 890	Ala	Glu	Val	Ser	Glu 895	Glu
40	Gly	Asp	Val	Gln 900	Leu	Asn	Pro	Ile	Phe 905	Pro	Phe	Asp	Phe	Lys 910	Leu	Gly
			,, _ ,					920					925			
1 5		Val 930					335					940				
50	,	Ile				750					955					960
		Val			<i>3</i> 03					970					975	
55	Leu			780					985					990		
			,,,					1000	,				1005			
0		Val 1010					1015	1				1020)			
5	Asn 1025					1030					1035					1040
	Asn				1045					1050					1055	
0	Gly	Leu	Ile	Thr 1060	Ser .	Asp	Ser	Asp	Asn 1065	Ile	Asn	Met	Trp	Ile 1070		Asp

	Phe Tyr Ile Phe Ala Lys Glu Leu Asp G 1075 1080	ly Lys Asp Ile Asn Ile Leu 1085
5	Phe Asn Ser Leu Gln Tyr Thr Asn Val V 1090 1095	al Lys Asp Tyr Trp Gly Asn
	Asp Leu Arg Tyr Asn Lys Glu Tyr Tyr M	et Val Asn Ile Asp Tyr Leu 1115 1120
10	The state of the s	
	Arg Asn Asn Asp Phe Asn Glu Gly To	2233
15	Ile Arg Gly Asn Thr Asn Asp Thr Arg Va	i i
20	Tyr Phe Asp Met Thr Ile Asn Asn Lys A:	
	Asn Glu Thr Met Tyr Ala Asp Asn His Se	er Thr Glu Asp Ile Tyr Ala
25	Ile Gly Leu Arg Glu Gln Thr Lys Asp Il	
	Gln Ile Gln Pro Met Asn Asn Thr Tyr Ty	
30	Lys Ser Asn Phe Asn Gly Glu Asn Ile Se	
35	Thr Tyr Arg Phe Arg Leu Gly Gly Asp Tr	
	1250 1255 Val Pro Thr Val Lys Gln Gly Asn Tyr Al	1260 a Ser Leu Leu Glu Ser Thr
40	Ser Thr His Trp Gly Phe Val Pro Val Se	1275 1280
	1285 12 (2) INFORMATION FOR SEQ ID NO:61:	90
45	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 1502 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: double	
50	(D) TOPOLOGY: linear	
55	<pre>(ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE:</pre>	
	(xi) SEQUENCE DESCRIPTION: SEQ ID	NO:61:
50	AGATCTCGAT CCCGCGAAAT TAATACGACT CACTA	· · · · · · · · · · · · · · · · · · ·
	TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGA	AGGAG ATATACC ATG GGC CAT 110 Met Gly His 1
55	CAT CAT CAT CAT CAT CAT CAT CAC AG His His His His His His His Se 5	C AGC GGC CAT ATC GAA GGT
70	CGT CAT ATG GCT AGC ATG GCT TTA TTA AA Arg His Met Ala Ser Met Ala Leu Leu Ly	A GAT ATA ATT AAT GAA TAT 212 s Asp Ile Ile Asn Glu Tyr

	. 20)				25	;				3()				35	
5					40)		шуз	> 116	45	Sei	Lei	ı Glr	Asr	Arg 50	A AAA Lys	260
10				55			361	GI	60	ASN	Ala	Glu	ı Val	Ser 65	Glu	GAA Glu	308
		•	70			AJII	710	75	Pne	Pro	Phe	Asp	Phe 80	Lys	Leu	GGT	356
15		85	•				90	Lys	val	116	vaı	7hr 95	Gln	Asn	Glu	AAT Asn	404
20	100		-		-	105		GIU	ser	Pne	llo	Ile	Ser	Phe	Trp	ATT Ile 115	452
25			AAT Asn	-,-	120	Vu_	561	Vell	Leu	125	GIA	Tyr	Thr	Ile	11e 130	Asp	500
30			AAA Lys	135		501	GIY	пр	140	iie	GIY	He	Ile	Ser 145	Asn	Phe	548
35			TTT Phe 150	••••	Leu	Dys	GIN	155	GIU	Asp	Ser	Glu	Gln 160	Ser	Ile	Asn	596
		165	TAT Tyr			361	170	ASII	Ala	Pro	GIÀ	Tyr 175	Asn	Lys	Trp	Phe	644
40	180		ACT Thr	v u1	****	185	ASII	Met	мет	GIY	190	Met	Lys	Ile	Tyr	Ile 195	692
45		1	AAA Lys	Deu	200	nsp	1111	iie	rys	Va1 205	Lys	Glu	Leu	Thr	Gly 210	Ile	740
50	AAT Asn			215	• • • • •	116	1111	ene	220	11e	Asn	Lys	Ile	Pro 225	Asp	Thr	788
55	GGT		230	••••	261	ASP	ser	235	Asn	lie	Asn	Met	Trp 240	Ile	Arg	Asp	836
5.5		245				Lys	250	Leu	ASP	GIY	Lys	Asp 255	lle	Asn	Ile	Leu	884
60	Phe 260					265		ASII	vaı	vai	Lуs 270	Asp	Tyr	Trp	Gly	Asn 275	932
65	GAT Asp				280	Lys .	JIU	TYE	ryr	мет 285	Val	Asn	Ile	Asp	Tyr 290	Leu	980
70	AAT A	AGA Arg	- / -	ATG ' Met ' 295	TAT (GCG A	AAC ' Asn :	TCA Ser	CGA Arg 300	CAA . Gln	ATT Ile	GTT Val	Phe .	AAT Asn 305	ACA Thr	CGT Arg	1028

	AGA Arg	AAT Asn	AAT Asn 310	MS11	GAC Asp	TTC Phe	AAT Asn	GAA Glu 315	ı Gıy	TAT Tyr	AAA Lys	ATT	ATA Ile 320	ATA Ile	AAA Lys	AGA Arg	1	076
. 5	ATC Ile	AGA Arg 325	GIY	AAT Asn	ACA Thr	AAT Asn	GAT Asp 330	Thr	AGA Arg	GTA Val	CGA Arg	GGA Gly 335	GGA Gly	GAT Asp	ATT Ile	TTA Leu	1:	124
10	340	1116	ASP	Mec	1111	345	ASN	Asn	Lys	Ala	Туг 350	Asn	Leu	Phe	Met	355	11	172
15	7,511	GIU	1111	Mec	360	GCA Ala	Asp	Asn	HIS	Ser 365	Thr	Glu	Asp	Ile	Tyr 370	Ala	12	220
20	•••	Oly	Deu	375	Gru	CAA Gln	inr	rys	380	lie	Asn	Asp	Asn	11e 385	Ile	Phe	12	868
125	0111	116	390	PIO	мес	AAT Asn	Asn	395	Tyr	тут	Tyr	Ala	Ser 400	Gln	Ile	Phe	13	16
125	2,3	405	ASII	Pile	ASI	GGA Gly	410	Asn	lle	Ser	Gly	Ile 415	Cys	Ser	lle	Gly	13	64
30	420	. 7 .	Arg	Pne	Arg	CTT Leu 425	GIY	GIY	Asp	Trp	Tyr 430	Arg	His	Asn	Tyr	Leu 435	14	12
35	VG	210	1111	vai	440	CAA Gln	GIY	Asn	Tyr	A1a 445	Ser	Leu	Leu	Glu	TCA Ser 450	ACA Thr	14	60
40	ser	inr	His	Trp 455	Gly	T TT Phe	Val	Pro	Val 460	AGT Ser	GAA Glu	AAAT	AGCT	Т			15	02
	(2)					SEQ												
45		(i) S	(A) (B)	LEN TYP	CHAR. GTH: E: a: OLOG	462 mino	amı aci	no a d	cids								
		(i	i) M	OLEC	ULE	TYPE	: pr	otei	n									
50						DESCI												
55	Met 1				3					10			•		15			
	Ile			20					25					30				
60	Asn (Glu	Tyr :	Phe i	Asn /	Asn 1	lle i	Asn A	Asp :	Ser :	Lys 1	lle 1	eu S 45	Ser I	Leu (Gln		
	Asn A	Arg : 50	Lys i	Asn :	Thr I	Leu 1	/al / 55	Asp '	Thr s	Ser (Gly T	Гуг <i>I</i> 60	Asn A	la c	Slu '	Val		
65	Ser (Glu (Glu (Gly A	Asp V	/al 0 70	Sln I	Leu /	Asn I	Pro :	Ile E 75	Phe F	ro P	he A	ap 1	Phe 80		
	Lys I	Leu (Gly s	Ser S	Ser (Gly G	Glu A	Asp A	Arg (3ly 1 90	Lys V	/al I	le V	al T	hr (Sln		
70	Asn (3lu /	Asn 1	le v	/al 1	yr A	sn S	Ger N	1et 1	ſyr (Slu S	er F	he s	er I	le s	Ser		

				100					105					110		
5	Phe	Trp	Ile 115	Arg	Ile	Asn	Lys	Trp 120	Val	Ser	Asn	Leu	Pro 125	Gly	туг	Th
	Ile	Ile 130	Asp	Ser	Val	Lys	Asn 135	Asn	Ser	Gly	Trp	Ser 140	Ile	Gly	Ile	Ile
10	Ser 145	Asn	Phe	Leu	Val	Phe 150	Thr	Leu	Lys	Gln	Asn 155	Glu	Asp	Ser	Glu	Glr 160
		Ile			162					170					175	
15	Lys	Trp	Phe	Phe 180	Val	Thr	Val	Thr	Asn 185	Asn	Met	Met	Gly	Asn 190	Met	Lys
20	Ile	Tyr	Ile 195	Asn	Gly	Lys	Leu	Ile 200	Asp	Thr	Ile	Lys	Val 205	Lys	Glu	Let
	Thr	Gly 210	Ile	Asn	Phe	Ser	Lys 215	Thr	Ile	Thr	Phe	Glu 220	Ile	Asn	Lys	Ile
25	Pro 225	Asp	Thr	Gly	Leu	11e 230	Thr	Ser	Asp	Ser	Asp 235	Asn	Ile	λsn	Met	Trp 240
	Ile	Arg	Asp	Phe	Tyr 245	Ile	Phe	Ala	Lys	Glu 250	Leu	Asp	Gly	Lys	Asp 255	Ile
30	Asn	Ile	Leu	Phe 260	Asn	Ser	Leu	Gln	Tyr 265	Thr	Asn	Val	Val	Lys 270	Asp	Tyr
35	Trp	Gly	Asn 275	Asp	Leu	Arg	Tyr	Asn 280	Lys	Glu	Tyr	Tyr	Met 285	Val	Asn	Ile
	Asp	Tyr 290	Leu	Asn	Arg	Tyr	Met 295	Tyr	Ala	Asn	Ser	Arg 300	Gln	Ile	Val	Phe
40	Asn 305	Thr	Arg	Arg	Asn	Asn 310	Asn	Asp	Phe	Asn	Glu 315	Gly	Tyr	Lys	Ile	11e 320
		Lys			325					330					335	-
45	Asp	Ile	Leu	Tyr 340	Phe	Asp	Met	Thr	Ile 345	Asn	Asn	Lys	Ala	Tyr 350	Asn	Leu
50	Phe	Met	Lys 355	Asn	Glu	Thr	Met	Tyr 360	Ala	Asp	Asn	His	Ser 365	Thr	Glu	Asp
	Ile	Tyr 370	Ala	Ile	Gly	Leu	Arg 375	Glu	Gln	Thr		Asp 380		Asn	qaA	Asn
55	11e 385	Ile	Phe	Gln	Ile	Gln 390	Pro	Met	Asn	Asn	Thr 395	Tyr	Tyr	Tyr	Ala	Ser 400
	Gln	Ile	Phe	Lys	Ser 405	Asn	Phe	Asn	Gly	Glu 410	Asn	Ile	Ser	Gly	Ile 415	Сув
60	Ser	Ile	Gly	Thr 420	Tyr	Arg	Phe	Arg	Leu 425	Gly	Gly	Asp	Trp	Tyr 430	Arg	His
65	Asn	Tyr	Leu 435	Val	Pro	Thr	Val	Lys 440	Gln	Gly	Asn	Tyr	Ala 445	Ser	Leu	Leu
	Glu	Ser 450	Thr	Ser	Thr	His	Trp 455	Gly	Phe	Val	Pro	Val 460	Ser	Glu		
70	(2)	INFO	RMAT	NOI	FOR	SEQ	ID N	10 : 6 3	:							

5	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 32 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA"</pre>	
10	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:	
	CGCCATGGCT TTATTAAAAG ATATAATTAA TG	3 ;
15	(2) INFORMATION FOR SEQ ID NO:64:	-
20	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 32 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
	<pre>(ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA"</pre>	
25	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:	
	GCAAGCTTTT ATTCACTTAC AGGTACAAAA CC	32
30	(2) INFORMATION FOR SEQ ID NO:65:	
. .	 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 3831 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: double 	
35	(D) TOPOLOGY: linear	
40	<pre>(ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: CDS</pre>	
	(B) LOCATION: 13828 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:	
45	ATG ACA TGG CCA GTA AAA GAT TTT AAT TAT AGT GAT CCT GTT AAT CAG	48
	1 11 11 11 11 11 11 11 11 11 11 11 11 1	
50	AAT GAT ATA TTA TAT TTA AGA ATA CCA CAA AAT AAG TTA ATT ACT ACA Asn Asp Ile Leu Tyr Leu Arg Ile Pro Gln Asn Lys Leu Ile Thr Thr 20 25 30	96
55	CCT GTA AAA GCT TTT ATG ATT ACT CAA AAT ATT TGG GTA ATA CCA GAA Pro Val Lys Ala Phe Met Ile Thr Gln Asn Ile Trp Val Ile Pro Glu 35 40 45	144
50	AGA TTT TCA TCA GAT ACT AAT CCA AGT TTA AGT AAA CCG CCC AGA CCT Arg Phe Ser Ser Asp Thr Asn Pro Ser Leu Ser Lys Pro Pro Arg Pro 50 55 60	192
	ACT TCA AAG TAT CAA AGT TAT TAT GAT CCT AGT TAT TTA TCT ACT GAT Thr Ser Lys Tyr Gln Ser Tyr Tyr Asp Pro Ser Tyr Leu Ser Thr Asp 65 70 75 80	240
5	GAA CAA AAA GAT ACA TTT TTA AAA GGG ATT ATA AAA TTA TTT AAA AGA Glu Gln Lys Asp Thr Phe Leu Lys Gly Ile Ile Lys Leu Phe Lys Arg 85 90 95	288
0	ATT AAT GAA AGA GAT ATA GGA AAA AAA TTA ATA A	336

	·			100)				105	I I				110	1		
5	Gly	TCA Ser	CCT Pro 115	- 11C	ATG Met	GGA Gly	GAT Asp	TCA Ser 120	Ser	ACG Thr	CCT Pro	GAA Glu	GAT Asp	Thr	TTT Phe	GAT Asp	38
10	TTT Phe	ACA Thr 130	CGT	CAT His	ACT Thr	ACT Thr	AAT Asn 135	ile	GCA Ala	GTT Val	GAA Glu	AAG Lys 140	TTT Phe	GAA Glu	AAT Asn	GGT Gly	43
	AGT Ser 145		AAA Lys	GTA Val	ACA Thr	AAT Asn 150	ATT Ile	ATA Ile	ACA Thr	CCA Pro	AGT Ser 155	GTA Val	TTG Leu	ATA Ile	TTT Phe	GGA Gly 160	. 48
15	CCA Pro	CTT	CCT Pro	AAT Asn	ATA Ile 165	TTA Leu	GAC Asp	TAT Tyr	ACA Thr	GCA Ala 170	TCC Ser	CTT Leu	ACA Thr	TTG Leu	CAA Gln 175	GGA Gly	521
20	CAA Gln	CAA Gln	TCA Ser	AAT Asn 180	CCA Pro	TCA Ser	TTT Phe	GAA Glu	GGG Gly 185	TTT Phe	GGA Gly	ACA Thr	TTA Leu	TCT Ser 190	ATA Ile	CTA Leu	576
25	AAA Lys	GTA Val	GCA Ala 195	CCT Pro	GAA Glu	TTT Phe	TTG Leu	TTA Leu 200	ACA Thr	TTT Phe	AGT Ser	GAT Asp	GTA Val 205	ACA Thr	TCT Ser	AAT Asn	624
30	CAA Gln	AGT Ser 210	TCA Ser	GCT Ala	GTA Val	TTA Leu	GGC Gly 215	AAA Lys	TCT Ser	ATA Ile	TTT Phe	TGT Cys 220	ATG Met	GAT Asp	CCA Pro	GTA Val	672
	ATA Ile 225	GCT Ala	TTA Leu	ATG Met	CAT His	GAG Glu 230	TTA Leu	ACA Thr	CAT His	TCT Ser	TTG Leu 235	CAT His	CAA Gln	TTA Leu	TAT Tyr	GGA Gly 240	720
35	ATA Ile	AAT Asn	ATA Ile	CCA Pro	TCT Ser 245	GAT Asp	AAA Lys	AGG Arg	ATT Ile	CGT Arg 250	CCA Pro	CAA Gln	G TT Val	AGC Ser	GAG Glu 255	GGA Gly	768
4()	TTT Phe	TTC Phe	TCT Ser	CAA Gln 260	GAT Asp	GGA Gly	CCC Pro	AAC Asn	GTA Val 265	CAA Gln	TTT Phe	GAG Glu	GAA Glu	TTA Leu 270	TAT Tyr	ACA Thr	816
45	TTT Phe	GGA Gly	GGA Gly 275	TTA Leu	GAT Asp	GTT Val	GAA Glu	ATA Ile 280	ATA Ile	CCT Pro	CAA Gln	ATT Ile	GAA Glu 285	AGA Arg	TCA Ser	CAA Gln	864
50	TTA Leu	AGA Arg 290	GAA Glu	AAA Lys	GCA Ala	TTA Leu	GGT Gly 295	CAC His	TAT Tyr	AAA Lys	GAT Asp	ATA Ile 300	GCG Ala	AAA Lys	AGA Arg	CTT Leu	912
	AAT Asn 305	AAT Asn	ATT Ile	AAT Asn	AAA Lys	ACT Thr 310	ATT Ile	CCT Pro	TCT Ser	AGT Ser	TGG Trp 315	ATT Ile	AGT Ser	AAT Asn	ATA Ile	GAT Asp 320	960
55	AAA Lys	TAT Tyr	AAA Lys	AAA Lys	ATA Ile 325	TTT Phe	TCT Ser	GAA Glu	AAG Lys	TAT Tyr 330	AAT Asn	TTT Phe	GAT Asp	AAA Lys	GAT Asp 335	AAT Asn	1008
60	ACA Thr	GGA Gly	AAT Asn	TTT Phe 340	GTT Val	GTA Val	AAT Asn	ATT Ile	GAT Asp 345	AAA Lys	TTC Phe	AAT Asn	AGC Ser	TTA Leu 350	TAT Tyr	TCA Ser	1056

4	GAC Asp	TTG Leu	ACT Thr 355	Asn	GTT Val	ATG Met	TCA Ser	GAA Glu 360	Val	GTT Val	TAT Tyr	TCT Ser	TCG Ser 365	Glr	TAT	AAT Asn	1104
5	GTT Val	AAA Lys 370	Asn	AGG Arg	ACT Thr	CAT	TAT Tyr 375	Phe	TCA Ser	AGG Arg	CAT Hís	TAT Tyr 380	Leu	CCT	GTA Val	TTT Phe	1152
10	GCA Ala 385	Asn	ATA Ile	TTA Leu	GAT Asp	GAT Asp 390	AAT Asn	ATT	TAT	ACT Thr	ATA Ile 395	Arg	GAT Asp	GGT Gly	TTT Phe	AAT Asn 400	1200
15	TTA Leu	ACA Thr	AAT Asn	AAA Lys	GGT Gly 405	TTT Phe	AAT Asn	ATA Ile	GAA Glu	AAT Asn 410	Ser	GGT Gly	CAG Gln	AAT Asn	ATA Ile 415	GAA Glu	1248
20	Arg	Asn	Pro	420	Leu	CAA Gln	Lys	Leu	Ser 425	Ser	Glu	Ser	Val	Val 430	Asp	Leu	1296
	Pne	Inr	Lys 435	Val	Cys	TTA Leu	Arg	Leu 440	Thr	Lys	Asn	Ser	Arg 445	Asp	Asp	Ser	1344
1 25	Thr	450	Ile	Lys	Val	AAA Lys	Asn 455	Asn	Arg	Leu	Pro	Туг 460	Val	Ala	Asp	Lys	1392
30	465	Ser	lle	Ser	Gln	GAA Glu 470	Ile	Phe	Glu	Asn	Lys 475	Ile	Ile	Thr	Asp	Glu 480	1440
35	rnr	Asn	Val	GLn	Asn 485	TAT Tyr	Ser	Asp	Asn	Phe 490	Ser	Leu	Asp	Glu	Ser 495	Ile	1488
40	neu	Asp	Gly	500	Val	CCT Pro	Ile	Asn	Pro 505	Glu	Ile	Val	Asp	Pro 510	Leu	Leu	1536
15	Pro	Asn	Va1 515	Asn	Met	GAA Glu	Pro	Leu 520	Asn	Leu	Pro	Gly	Glu 525	Glu	Ile	Val	1584
45	Pne	17r 530	Asp	Asp	lle	ACT Thr	Lys 535	Tyr	Val	Asp	Tyr	Leu 540	Asn	Ser	Tyr	Tyr	1632
50	1yr 545	Leu	Glu	Ser	GIn	AAA Lys 550	Leu	Ser	Asn	Asn	Val 555	Glu	Asn	Ile	Thr	Leu 560	1680
55	inr	Inr	Ser	Val	565	GAA Glu	Ala	Leu	Gly	Tyr 570	Ser	Asn	Lys	Ile	Tyr 575	Thr	1728
60	Pne	Leu	Pro	Ser 580	Leu	GCT Ala	Glu	Lys	Val 585	Asn	Lys	Gly	Val	Gln 590	Ala	Gly	1776
	TTA Leu	TTC Phe	TTA Leu 595	AAT Asn	TGG Trp	GCG Ala	AAT Asn	GAA Glu 600	GTA Val	GTT Val	GAG Glu	GAT Asp	TTT Phe 605	ACT Thr	ACA Thr	AAT Asn	1824
65	TTE	ATG Met 610	AAG Lys	AAA Lys	GAT Asp	ACA Thr	TTG Leu 615	GAT Asp	AAA Lys	ATA Ile	TCA Ser	GAT Asp 620	GTA Val	TCA Ser	GTA Val	ATA Ile	1872
70	ATT Ile	CCA Pro	TAT Tyr	ATA Ile	GGA Gly	CCT Pro	GCC Ala	TTA Leu	AAT Asn	ATA Ile	GGA Gly	AAT Asn	TCA Ser	GCA Ala	TTA Leu	AGG Arg	1920

	625	5				630	;				639	5				640	
5	GGA Gly	AA7 Asr	TTT Phe	AAG Lys	Glr 645		TTI Phe	GC#	A ACA	GCT Ala 650	r GT?	C GTA Val	GCI Ala	TTI Phe	TTA Leu 655	TTA Leu	1968
10	GAG Glu	GG# Gly	TTI Phe	CCA Pro 660	Gru	TTT Phe	ACT Thr	ATA	CCT Pro	Ala	CTC Leu	GGT Gly	GTA Val	TT1 Phe 670	Thr	TTT Phe	2016
	TAT Tyr	AGT Ser	TCT Ser 675	-16	CAA Gln	GAA Glu	AGA Arg	GAG Glu 680	Lys	ATT	ATT	AAA Lys	ACT Thr 685	Ile	GAA Glu	AAT Asn	2064
15	TGT Cys	TTG Leu 690	GAA Glu	CAA Gln	AGA Arg	GTT Val	AAG Lys 695	AGA Arg	TGG Trp	AAA Lys	GAT Asp	TCA Ser 700	TAT Tyr	CAA Gln	TGG Trp	ATG Met	2112
20	GTA Val 705	TCA Ser	AAT Asn	TGG Trp	TTG Leu	TCA Ser 710	AGA Arg	ATT Ile	ACT Thr	ACT Thr	CAA Gln 715	TTT Phe	AAT Asn	CAT His	ATA Ile	AAT Asn 720	2160
25				TAT Tyr	725	261	Leu	ser	ryr	730	Ala	Asp	Ala	Ile	Lys 735	Ala	2208
30	-,-		пор	TTA Leu 740	GIU	Tyt	Lys	Lys	745	Ser	Gly	Ser	Asp	Lys 750	Glu	Asn	2256
		_,5	755	CAA Gln	val	Gru	ASN	760	Lys	Asn	Ser	Leu	Asp 765	Val	Lys	Ile	2304
35		770		ATG Met	ASII	ASI	775	ASN	FÀS	Pne	He	780	Glu	Cys	Ser	Val	2352
40	785	-,-	Deu	TTT Phe	БУБ	790	мес	ren	Pro	Lys	Val 795	Ile	Asp	Glu	Leu	Asn 800	2400
45	-,-		~2Þ	TTA Leu	805	1111	Lys	Inr	Glu	B10	Ile	Asn	Leu	Ile	Asp 815	Ser	2448
50		ASI	116	ATT Ile 820	Leu	vai	GIŸ	GIu	Val 825	Asp	Arg	Leu	Lys	Ala 830	Lys	Val	2496
			835	TTT Phe	GIU	ASII	mr	Met 840	Pro	Phe	Asn	Ile	Phe 845	Ser	Tyr	Thr	2544
55		B50	001	TTA Leu	Leu	цуѕ	855	rre	116	Asn	GLu	Tyr 860	Phe	Asn	Ser	Ile	2592
60	865	ЛЭР	Jei	AAA Lys	116	870	ser	Leu	GIn	Asn	Lys 875	rys	Asn	Ala	Leu	Val 880	2640
65	ПОР		561		885	ASII	АІА	GIu	Val	Arg 890	Val	Gly	Asp .	Asn	Val 895	Gln	2688
70	CTT .	AAT Asn	ACG Thr	ATA Ile 900	TAT Tyr	ACA Thr	AAT Asn	GAC Asp	TTT Phe 905	AAA Lys	TTA Leu	AGT Ser	Ser	TCA Ser	GGA (GAT Asp	2736

	AAJ Lys	A ATT	TATA FILE 915	val	A AAT . Asn	TTA Leu	AAT Asn	AAT Asn 920	Asn	T ATT	TTA Leu	TAT Tyr	AGC Ser 925	Ala	T ATT	TAT Tyr	2784
5	GAC Glu	AAC Asn 930	Ser	AGT Ser	GTT Val	AGT Ser	TTT Phe 935	Trp	ATT Ile	'AAG Lys	ATA Ile	TCT Ser 940	Lys	GA1 Asp	TTA Leu	ACT Thr	2832
10	AAT Asr 945	ı ser	CAT His	AAT Asn	GAA Glu	TAT Tyr 950	Thr	ATA Ile	ATT Ile	AAC Asn	AGT Ser 955	Ile	GAA Glu	CAA Glr	AAT Asn	TCT Ser 960	2880
15	GGG Gly	TGG Trp	AAA Lys	TTA Leu	TGT Cys 965	ATT Ile	AGG Arg	AAT Asn	GGC Gly	AAT Asn 970	Ile	GAA Glu	TGG Trp	ATT	TTA Leu 975	CAA Gln	2928
20	Asp	val	Asn	980	Lys	Tyr	Lys	Ser	Leu 985	Ile	Phe	Asp	Tyr	Ser 990	Glu		2976
·o -	Leu	ser	H1S 995	Thr	GGA Gly	Tyr	Thr	Asn 100	0 FÀR	Trp	Phe	Phe	Val 100	Thr 5	Ile	Thr	3024
25	AAT Asn	AAT Asn 101	ile	ATG Met	GGG Gly	TAT Tyr	ATG Met 101	Lys	CTT Leu	TAT Tyr	ATA Ile	AAT Asn 102	Gly	GAA Glu	TTA Leu	AAG Lys	3072
30	CAG Gln 102	Ser	CAA Gln	AAA Lys	ATT Ile	GAA Glu 1030	Asp	TTA Leu	GAT Asp	GAG Glu	GTT Val 103	Lys	TTA Leu	GAT Asp	AAA Lys	ACC Thr 1040	3120
35	He	Val	Phe	Gly	ATA Ile 1049	Asp	Glu	Asn	Ile	Asp 105	Glu O	Asn	Gln	Met	Leu 105!	Trp 5	3168
40	lle	Arg	Asp	Phe 106		Ile	Phe	Ser	Lys 106	Glu 5	Leu	Ser	Asn	Glu 107	Asp 0	Ile	3216
	Asn	lie	Val 1079	Tyr	GAG Glu	Gly	Gln	11e 108(Leu)	Arg	Asn	Val	11e 1089	Lys	Asp	Tyr	3264
45	тър	1090	Asn)	Pro	TTG Leu	Lys	Phe 1099	Asp	Thr	Glu	Tyr	Tyr 1100	lle	Ile	Asn	Asp	3312
50	110	5	TIE	Asp	AGG Arg	1110	lle	Ala	Pro	Glu	Ser 1115	Asn	Val	Leu	Val	Leu 1120	3360
55	vai	Arg	Tyr	Pro	GAT Asp 1125	Arg	Ser	Lys	Leu	Tyr 1130	Thr	Gly	Asn	Pro	11e	Thr	3408
60	ATT Ile	AAA Lys	TCA Ser	GTA Val 1140	TCT Ser	GAT Asp	AAG Lys	AAT Asn	CCT Pro 1145	Tyr	AGT Ser	AGA Arg	ATT Ile	TTA Leu 1150	Asn	GGA Gly	3456
	GAT Asp	AAT Asn	ATA Ile 1155	Ile	CTT Leu	CAT His	ATG Met	TTA Leu 1160	Tyr	AAT Asn	AGT Ser	AGG Arg	AAA Lys 1165	Tyr	ATG Met	ATA Ile	3504
65	ATA Ile	AGA Arg 1170	Asp	ACT Thr	GAT Asp	ACA Thr	ATA Ile 1 1 75	Tyr	GCA Ala	ACA Thr	CAA Gln	GGA Gly 1180	Gly	GAG Glu	TGT Cys	TCA Ser	3552
70	· CAA Gln	AAT Asn	TGT Cys	GTA Val	TAT Tyr	GCA Ala	TTA Leu	AAA Lys	TTA Leu	CAG Gln	AGT Ser	AAT Asn	TTA Leu	GGT Gly	AAT Asn	TAT Tyr	3600

															•
	1185			119	0				119	5				1200	
5	GGT ATA	GGT :	ATA TTT Ile Phe 120	Ser	ATA Ile	Lys	AAT Asn	ATT Ile 121	Val	TCT Ser	AAA Lys	TAA neA	AAA Lys 121	Tyr	3648
10	TGT AGT Cys Ser	GIII .	ATT TTC Ile Phe 1220	TCT Ser	AGT Ser	TTT Phe	AGG Arg 122	Glu	AAT Asn	ACA Thr	ATG Met	CTT Leu 123	Leu	GCA Ala	3696
	GAT ATA	TAT A Tyr 1	AAA CCT Lys Pro	TGG Trp	AGA Arg	TTT Phe 124	Ser	TTT Phe	AAA Lys	AAT Asn	GCA Ala 124	Tyr	ACG Tḩr	CCA Pro	3744
15	GTT GCA Val Ala 125	val	ACT AAT Thr Asn	TAT Tyr	GAA Glu 125	Thr	AAA Lys	CTA Leu	TTA Leu	TCA Ser 126	Thr	TCA Ser	TCT Ser	TTT Phe	3792
20	TGG AAA Trp Lys 1265	TTT A	ATT TCT lle Ser	AGG Arg 1270	Asp	CCA Pro	GGA Gly	TGG Trp	GTA Val 1279	Glu	TAA				3831
	(2) INF	ORMAT	ON FOR	SEQ	ID I	NO : 6	6 :								
25		(i) SE	EQUENCE (A) LEI (B) TY: (D) TO:	NGTH: PE: ā	: 12° amino	76 at	mino id	: acid	is						
30	(ii) MC	LECULE												
			QUENCE					QI Q	NO : 6	56:					
35	Met Thr	Trp F	ro Val	Lys	Asp	Phe	Asn	Tyr 10	Ser	Asp	Pro	Val	Asn 15	Asp	
	Asn Asp	Ile L	eu Tyr 20	Leu	Arg	Ile	Pro 25	Gln	Asn	Lys	Leu	Ile 30		Thr	
40	Pro Val	Lys A	la Phe	Met	Ile	Thr 40		Asn	Ile	Trp	Val 45		Pro	Glu	
45	Arg Phe	Ser S	er Asp	Thr	Asn 55	Pro	Ser	Leu	Ser	Lys 60	Pro	Pro	Arg	Pro	
	Thr Ser	Lys T	yr Gln	Ser 70	Tyr	Tyr	Asp	Pro	Ser 75	Tyr	Leu	Ser	Thr	Asp 80	
50	Glu Gin	Lys A	sp Thr 85	Phe	Leu	Lys	Gly	Ile 90	Ile	Lys	Leu	Phe	Lys 95	Arg	
	Ile Asn	Glu A	rg Asp	Ile	Gly	Lys	Lys 105	Leu	Ile	Asn	Туr	Leu 110	Val	Val	
55	Gly Ser	Pro P	he Met	Gly	Asp	Ser 120	Ser	Thr	Pro	Glu	A sp 125	Thr	Phe	Asp	
•	Phe Thr	Arg H	is Thr	Thr	Asn 135	Ile	Ala	Val	Glu	Lys 140	Phe	Glu	Asn	Gly	
60	Ser Trp 145	Lys V	al Thr	Asn 150	Ile	Ile	Thr	Pro	Ser 155		Leu	Ile	Phe	Gly 160	
65	Pro Leu	Pro A	sn Ile 165	Leu	Asp	Tyr	Thr	Ala 170	Ser	Leu	Thr	Leu	Gln 175	Gly	
	Gln Gln	Ser A	sn Pro 80	Ser	Phe	Glu	Gly 185	Phe	Gly	Thr	Leu	Ser 190	Ile	Leu	
70	Lys Val	Ala P	ro Glu	Phe	Leu	Leu	Thr	Phe	Ser	Asp	Val	Thr	Ser	Asn	

195 200 205 Gln Ser Ser Ala Val Leu Gly Lys Ser Ile Phe Cys Met Asp Pro Val 220 5 Ile Ala Leu Met His Glu Leu Thr His Ser Leu His Gln Leu Tyr Gly Ile Asn fle Pro Ser Asp Lys Arg Ile Arg Pro Gln Val Ser Glu Gly 10 Phe Phe Ser Gln Asp Gly Pro Asn Val Gln Phe Glu Glu Leu Tyr Thr 15 Phe Gly Gly Leu Asp Val Glu Ile Ile Pro Gln Ile Glu Arg Ser Gln Leu Arg Glu Lys Ala Leu Gly His Tyr Lys Asp Ile Ala Lys Arg Leu 20 Asn Asn Ile Asn Lys Thr Ile Pro Ser Ser Trp Ile Ser Asn Ile Asp Lys Tyr Lys Lys Ile Phe Ser Glu Lys Tyr Asn Phe Asp Lys Asp Asn 25 Thr Gly Asn Phe Val Val Asn Ile Asp Lys Phe Asn Ser Leu Tyr Ser 30 Asp Leu Thr Asn Val Met Ser Glu Val Val Tyr Ser Ser Gln Tyr Asn 360 Val Lys Asn Arg Thr His Tyr Phe Ser Arg His Tyr Leu Pro Val Phe 370 380 35 Ala Asn Ile Leu Asp Asp Asn Ile Tyr Thr Ile Arg Asp Gly Phe Asn 395 Leu Thr Asn Lys Gly Phe Asn Ile Glu Asn Ser Gly Gln Asn Ile Glu 40 410 Arg Asn Pro Ala Leu Gln Lys Leu Ser Ser Glu Ser Val Val Asp Leu 45 Phe Thr Lys Val Cys Leu Arg Leu Thr Lys Asn Ser Arg Asp Asp Ser Thr Cys Ile Lys Val Lys Asn Asn Arg Leu Pro Tyr Val Ala Asp Lys 50 Asp Ser Ile Ser Gln Glu Ile Phe Glu Asn Lys Ile Ile Thr Asp Glu Thr Asn Val Gln Asn Tyr Ser Asp Asn Phe Ser Leu Asp Glu Ser Ile 55 Leu Asp Gly Gln Val Pro Ile Asn Pro Glu Ile Val Asp Pro Leu Leu 505 60 Pro Asn Val Asn Met Glu Pro Leu Asn Leu Pro Gly Glu Glu Ile Val Phe Tyr Asp Asp Ile Thr Lys Tyr Val Asp Tyr Leu Asn Ser Tyr Tyr 65 Tyr Leu Glu Ser Gln Lys Leu Ser Asn Asn Val Glu Asn Ile Thr Leu Thr Thr Ser Val Glu Glu Ala Leu Gly Tyr Ser Asn Lys Ile Tyr Thr 70

,	Ph€	e Lei	u Pro	Ser 580	Leu)	Ala	a Glu	ı Ly:	s Va 58	l As 5	n Ly	s Gl	y Va	1 Gl: 59	n Al	a Gly
5	Leu	ı Phe	E Let 599	Asn	Trp	Ala	a Asr	Glu 600	u Vai	l Va	1 G1:	u Ası	Ph 60	e Th:	r Th	r Asn
•	Ile	Met 610	Lys	5 Lys	Asp	Thr	Leu 615	ı Asp	Ly:	s Ile	e Sei	r Ası 620	o Va	l Sei	r Va	l Ile
10	11e 625	Pro	Туг	Ile	Gly	Pro 630	Ala	Leu	ı Asr	ı Ile	∈ Gl ₃ 635	/ Asr	ı Se	r Ala	Lei	Arg 640
15	Gly	Asn	Phe	Lys	Gln 645	Ala	Phe	Ala	Thr	Ala 650	a Gly	/ Val	Alá	a Phe	Lev 655	ı Leu
	Glu	Gly	Phe	Pro 660	Glu	Phe	Thr	Ile	Pro 665	Ala	Leu	Gly	Va]	Phe 670	Thr	Phe
20	Tyr	Ser	Ser 675	Ile	Gln	Glu	Arg	Glu 680	Lys	Ile	lle	Lys	Th:	Ile	Glu	Asn
				Gln			0,5					700				
25	Val 705	Ser	Asn	Trp	Leu	Ser 710	Arg	Ile	Thr	Thr	Gln 715	Phe	Asn	His	Ile	Asn 720
30				Tyr						/30					735	
				Leu 740					743					750		
35				Gln				, 00					765			
10				Met			,					780				
40				Phe							795					800
45										810					815	
				Ile 820					825					830		
50	Asn							340					845			
55				Leu :			933					860				
JJ				Lys							8/5					880
60	Asp 1			`	505					890					895	
	Leu A			,,,,					905					910		
65	Lys :							920					925			
70						•	,,,					940				
70	Asn S	ser i	His A	Asn (Slu 7	`yr `	Thr :	Ile :	Ile .	Asn	Ser	Ile	Glu	Gln .	Asn	Ser

	945	i				950					955					960
,	Gly	, Trp	Lys	Leu	Cys	Ile	Arg	Asn	Gly	Asn			Tro	Ile	Leu	
5					965					970					975	
			Asn	980					985					990		
10	Leu	Ser	His 995	Thr	Gly	Tyr	Thr	Asn 100	Lys 0	Trp	Phe	Phe	Val 100		Ile	Thr
	Asn	Asn 101	Ile O	Met	Gly	Tyr	Met 101	Lys 5	Leu	Tyr	Ile	Asn 102		Glu	Leu	Lys
15	Gln 102	Ser 5	Gln	Lys	Ile	Glu 103	Asp 0	Leu	Asp	Glu	Val 103		Leu	Asp	Lys	Thr
20	Ile	Val	Phe	Gly	Ile 1049	Asp 5	Glu	Asn	Ile	Asp 105	Glu D	Asn	Gln	Met	Leu 105	
	Ile	Arg	Asp	Phe 1060	Asn O	Ile	Phe	Ser	Lys 106	Glu 5	Leu	Ser	Asn	Glu 107		Ile
25	Asn	Ile	Val 1079	Tyr	Glu	Gly	Gln	Ile 1080	Leu)	Arg	Asn	Val	Ile 1089		Asp	Туг
	Trp	Gly 109	Asn 0	Pro	Leu	Lys	Phe 1099	Asp	Thr	Glu	Tyr	Tyr		Ile	Asn	Asp
30	Asn 110	Tyr 5	Ile	Asp	Arg	Tyr 1110	Ile	Ala	Pro	Glu	Ser 111!		Val	Leu	Val	Leu 1120
35	Val	Arg	Tyr	Pro	Asp 1125	Arg	Ser	Lys	Leu	Tyr 1130	Thr	Gly	Asn	Pro	Ile 1135	
	Ile	Lys	Ser	Val 1140	Ser	Asp	Lys	Asn	Pro 1145	Tyr	Ser	Arg	Ile	Leu 1150		Gly
4()	Asp	Asn	Ile 1155	Ile	Leu	His	Met	Leu 1160		Asn	Ser	Arg	Lys 1165		Met	Ile
	Ile	Arg 117	Asp O	Thr	Asp	Thr	Ile 1175	Tyr	Ala	Thr	Gln	Gly 1180		Glu	Cys	Ser
45	Gln 1189	Asn 5	Cys	Val	Tyr	Ala 1190	Leu)	Lys	Leu	Gln	Ser 1199	Asn	Leu	Gly	Asn	Tyr 1200
50	Gly	Ile	Gly	Ile	Phe 1205	Ser	Ile	Lys	Asn	Ile 1210		Ser	Lys	Asn	Lys 1215	
	Cys	Ser	Gln	Ile 1220	Phe	Ser	Ser	Phe	Arg 1225	Glu	Asn	Thr	Met	Leu 1230		Ala
55	Asp	Ile	Tyr 1235	Lys	Pro	Trp	Arg	Phe 1240	Ser	Phe	Lys	Asn	Ala 1245		Thr	Pro
	Val	Ala 1250	Val	Thr	Asn	Tyr	Glu 1255	Thr	Lys	Leu	Leu	Ser 1260		Ser	Ser	Phe
50	Trp 1265	Lys	Phe	Ile	Ser	Arg 1270	Asp	Pro	Gly		Val 1275					

- 351 -

	(2)	INI	FORM	ATIO	1 FOI	R SE	Q ID	NO:	67:								
5		(:		EQUEN (A) I (B) I (C) S (D) I	LENGT TYPE : TRAN	TH: 1 DEDN	1469 Cleic NESS:	base ac: do:	e pa: id	irs							٠
10		(ii) MC	DLECU	JLE 1	YPE:	DNA	A (ge	enomi	ic)							
		· (i)	1	EATUR (A) N (B) L	IAME/	KEY:	CDS	; 314	160								
15		(xi) SE	QUEN	CE D	ESCR	IPTI	ON:	SEQ	ID N	10 : 67	' :			,		
	AGA	TCTC	GAT	CCCG	CGAA	AT T	'AATA	CGAC	T CA	CTAT	' A GGG	GAA	TTGT	'GAG	CGGA	TAACA	A 6
20														ATG	GGC Gly	CAT His	11
25	CAT His	CAT His		CAT His	CAT His	CAT	CAT His	HIS	CAC His	AGC Ser	AGC Ser	GGC Gly 15	His	ATC Ile	GAA Glu	GGT	16
30	20		nec	Ala	261	25	АІА	Leu	Leu	Lys	Asp 30	Ile	Ile	Asn	Glu	35	21
	TTC Phe	AAT Asn	AGT Ser	ATT Ile	AAT Asn 40	GAT Asp	TCA Ser	AAA Lys	ATT Ile	TTG Leu 45	AGC Ser	TTA Leu	CAA Gln	AAC Asn	AAA Lys 50		26
35	AAT Asn	GCT Ala	TTA Leu	GTG Val 55	GAT Asp	ACA Thr	TCA Ser	GGA Gly	TAT Tyr 60	AAT Asn	GCA Ala	GAA Glu	GTG Val	AGG Arg 65	GTA Val	GGA Gly	308
40	GAT Asp	AAT Asn	GTT Val 70	CAA Gln	CTT Leu	AAT Asn	ACG Thr	ATA Ile 75	Tyr	ACA Thr	AAT Asn	GAC Asp	TTT Phe 80	AAA Lys	TTA Leu	AGT Ser	356
45	AGT Ser	TCA Ser 85	GGA Gly	GAT Asp	AAA Lys	ATT Ile	ATA Ile 90	GTA Val	AAT Asn	TTA Leu	AAT Asn	AAT Asn 95	AAT Asn	ATT Ile	TTA Leu	TAT Tyr	404
50	AGC Ser 100	GCT Ala	ΛTT Ile	TAT Tyr	GAG Glu	AAC Asn 105	TCT Ser	AGT Ser	GTT Val	AGT Ser	TTT Phe 110	TGG Trp	ATT Ile	AAG Lys	ATA Ile	TCT Ser 115	452
	AAA Lys	GAT Asp	TTA Leu	ACT Thr	AAT Asn 120	TCT Ser	CAT His	AAT Asn	GAA Glu	TAT Tyr 125	ACA Thr	ATA Ile	ATT Ile	AAC Asn	AGT Ser 130	ATA Ile	500
55	GAA Glu	CAA Gln	AAT Asn	TCT Ser 135	GGG Gly	TGG Trp	AAA Lys	TTA Leu	TGT Cys 140	ATT Ile	AGG Arg	AAT Asn	GGC Gly	AAT Asn 145	ATA Ile	GAA Glu	546
60	TGG Trp	ATT Ile	TTA Leu 150	CAA Gln	GAT Asp	GTT Val	AAT Asn	AGA Arg 155	AAG Lys	TAT Tyr	AAA Lys	AGT Ser	TTA Leu 160	ATT Ile	TTT Phe	GAT Asp	596
65	TAT Tyr	AGT Ser 165	GAA Glu	TCA Ser	TTA Leu	AGT Ser	CAT His 170	ACA Thr	GGA Gly	TAT Tyr	ACA Thr	AAT Asn 175	AAA Lys	TGG Trp	TTT Phe	TTT Phe	644
70	GTT Val 180	ACT Thr	ATA Ile	ACT Thr	AAT Asn	AAT Asn 185	ATA Ile	ATG Met	GGG Gly	TAT Tyr	ATG Met 190	AAA Lys	CTT Leu	TAT Tyr	ATA Ile	AAT Asn 195	692

	GGA Gly	GAA Glu	TTA Leu	AAG Lys	Gln 200	Ser	CAA Gln	AAA Lys	ATT Ile	GAA Glu 205	. Asp	TTA Leu	GAT Asp	GAC	GTT Val 210	AAG Lys	740
5	TTA Leu	GAT Asp	AAA Lys	ACC Thr 215	11e	GTA Val	TTT Phe	GGA Gly	ATA Ile 220	Asp	GAG Glu	AAT Asn	ATA Ile	GAT Asp 225	Glu	AAT Asn	788
10	C AG Gln	ATG Met	CTT Leu 230	Trp	ATT Ile	AGA Arg	GAT Asp	TTT Phe 235	Asn	ATT Ile	TTT Phe	TCT Ser	AAA Lys 240	Glu	TTA Leu	AGT Ser	836
15	AAT Asn	GAA Glu 245	ASP	ATT Ile	AAT Asn	ATT Ile	GTA Val 250	Tyr	GAG Glu	GGA Gly	CAA Gln	ATA Ile 255	TTA Leu	AGA Arg	AAT Asn	GTT Val	884
20	ATT Ile 260	AAA Lys	GAT Asp	TAT Tyr	TGG Trp	GGA Gly 265	AAT Asn	CCT Pro	TTG Leu	AAG Lys	TTT Phe 270	Asp	ACA Thr	GAA Glu	TAT Tyr	TAT Tyr 275	932
	ATT Ile	ATT Ile	AAT Asn	GAT Asp	AAT Asn 280	TAT Tyr	ATA Ile	GAT Asp	AGG Arg	TAT Tyr 285	ATT Ile	GCA Ala	CCT Pro	GAA Glu	AGT Ser 290	AAT Asn	980
25	GTA Val	CTT Leu	GTA Val	CTT Leu 295	GTT Val	CGG Arg	TAT Tyr	CCA Pro	GAT Asp 300	AGA Arg	TCT Ser	AAA Lys	TTA Leu	TAT Tyr 305	ACT Thr	GGA Gly	1028
30	AAT Asn	CCT Pro	ATT Ile 310	ACT Thr	ATT Ile	AAA Lys	TCA Ser	GTA Val 315	TCT Ser	GAT Asp	AAG Lys	AAT Asn	CCT Pro 320	TAT Tyr	AGT Ser	AGA Arg	1076
35	ATT Ile	TTA Leu 325	AAT Asn	GGA Gly	GAT Asp	AAT Asn	ATA Ile 330	ATT Ile	CTT Leu	CAT His	ATG Met	TTA Leu 335	TAT Tyr	AAT Asn	AGT Ser	AGG Arg	1124
40	AAA Lys 340	TAT Tyr	ATG Met	ATA Ile	ATA Ile	AGA Arg 345	GAT Asp	ACT Thr	GAT Asp	ACA Thr	ATA Ile 350	TAT Tyr	GCA Ala	ACA Thr	CAA Gln	GGA Gly 355	1172
	GGA Gly	GAG Glu	TGT Cys	TCA Ser	CAA Gln 360	AAT Asn	TGT Cys	GTA Val	TAT Tyr	GCA Ala 365	TTA Leu	AAA Lys	TTA Leu	CAG Gln	AGT Ser 370	AAT Asn	1220
45	TTA Leu	GGT Gly	AAT Asn	TAT Tyr 375	GGT Gly	ATA Ile	GG T Gly	ATA Ile	TTT Phe 380	AGT Ser	ATA Ile	AAA Lys	AAT Asn	ATT Ile 385	GTA Val	TCT Ser	1268
50	AAA Lys	TAA Asn	AAA Lys 390	TAT Tyr	TGT Cys	AGT Ser	CAA Gln	ATT Ile 395	TTC Phe	TCT Ser	AGT Ser	TTT Phe	AGG Arg 400	GAA Glu	AAT Asn	ACA Thr	1316
55	ATG Met	CTT Leu 405	CTA Leu	GCA Ala	GAT Asp	IIe	TAT Tyr 410	AAA Lys	CCT Pro	TGG Trp	AGA Arg	TTT Phe 415	TCT Ser	TTT Phe	AAA Lys	AAT Asn	1364
60	GCA Ala 420	TAC Tyr	ACG Thr	CCA Pro	GTT Val	GCA Ala 425	GTA Val	ACT Thr	AAT Asn	TAT Tyr	GAA Glu 430	ACA Thr	AAA Lys	CTA Leu	TTA Leu	TCA Ser 435	1412
	ACT Thr	TCA Ser	TCT Ser	Pne	TGG Trp 440	AAA Lys	TTT Phe	ATT Ile	Ser	AGG Arg 445	GAT Asp	CCA Pro	GGA Gly	TGG Trp	GTA Val 450	GAG Glu	1460
65	AAAT	AGCT	T														1469

(2) INFORMATION FOR SEQ ID NO:68:

70

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 451 amino acids

(B) TYPE: amino acid(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein 5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:68: Met Gly His His His His His His His His Ser Ser Gly His 10 Ile Glu Gly Arg His Met Ala Ser Met Ala Leu Leu Lys Asp Ile Ile Asn Glu Tyr Phe Asn Ser Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln $\frac{35}{40}$ 15 Asn Lys Lys Asn Ala Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val 20 Arg Val Gly Asp Asn Val Gln Leu Asn Thr Ile Tyr Thr Asn Asp Phe 65 70 75 80 Lys Leu Ser Ser Ser Gly Asp Lys Ile Ile Val Asn Leu Asn Asn Asn 25. Ile Leu Tyr Ser Ala Ile Tyr Glu Asn Ser Ser Val Ser Phe Trp Ile Lys Ile Ser Lys Asp Leu Thr Asn Ser His Asn Glu Tyr Thr Ile Ile 30 Asn Ser Ile Glu Gln Asn Ser Gly Trp Lys Leu Cys Ile Arg Asn Gly 135 35 Asn Ile Glu Trp Ile Leu Gln Asp Val Asn Arg Lys Tyr Lys Ser Leu Ile Phe Asp Tyr Ser Glu Ser Leu Ser His Thr Gly Tyr Thr Asn Lys 40 Trp Phe Phe Val Thr Ile Thr Asn Asn Ile Met Gly Tyr Met Lys Leu 185 Tyr Ile Asn Gly Glu Leu Lys Gln Ser Gln Lys Ile Glu Asp Leu Asp 45 Glu Val Lys Leu Asp Lys Thr Ile Val Phe Gly Ile Asp Glu Asn Ile 215 Asp Glu Asn Gln Met Leu Trp Ile Arg Asp Phe Asn Ile Phe Ser Lys 50 Glu Leu Ser Asn Glu Asp Ile Asn Ile Val Tyr Glu Gly Gln Ile Leu 55 Arg Asn Val Ile Lys Asp Tyr Trp Gly Asn Pro Leu Lys Phe Asp Thr Glu Tyr Tyr Ile Ile Asn Asp Asn Tyr Ile Asp Arg Tyr Ile Ala Pro 60 Glu Ser Asn Val Leu Val Leu Val Arg Tyr Pro Asp Arg Ser Lys Leu 65 Tyr Thr Gly Asn Pro Ile Thr Ile Lys Ser Val Ser Asp Lys Asn Pro Tyr Ser Arg Ile Leu Asn Gly Asp Asn Ile Ile Leu His Met Leu Tyr

70

	Asn	Ser	Arg	Lys 340	Tyr	Met	Ile	: Ile	Arg 345	Asp	Thr	Asp	Thr	11e		r Ala	•
5	Thr	Gln	Gly 355	Gly	Glu	Cys	Ser	Gln 360	Asn	Cys	Val	туг	Ala 365		Lys	. Leu	
	Gln	Ser 370	Asn	Leu	Gly	Asn	Tyr 375	Gly	Ile	Gly	Ile	Phe 380		Ile	Lys	. Asn	
10	Ile 385	Val	Ser	Lys	Asn	Lys 390	Tyr	Cys	Ser	Gln	Ile 395		Ser	Ser	Phe	Arg 400	
15	Glu	Asn	Thr	Met	Leu 405	Leu	Ala	Asp	Ile	Tyr 410		Pro	Trp	Arg	Phe 415	Ser	
-	Phe	Lys	Asn	Ala 420	Tyr	Thr	Pro	Val	Ala 425	Val	Thr	Asn	Tyr	Glu 430		Lys	
20	Leu	Leu	Ser 435	Thr	Ser	Ser	Phe	Trp 440	Lys	Phe	Ile	Ser	Arg	Asp	Pro	Gly	
	Trp	Val 450	Glu														
25	(2)	INFO	ORMAT	rion	FOR	SEQ	ID I	NO : 6	9 :								
30		(i)	(E	A) LI B) TY C) ST	ENGTI (PE : [RANI	IARAC I: 32 nucl DEDNE DGY:	2 bas Leic ESS:	se pa acio sing	airs d								
35				() DE	ESCRI	PTIC)N: ,	/des	c = '	"DNA	"						
	GCA	AGCTI	SEC							TD NO):69	:					
40	(2)		RMAT														32
45		(i)	(B	l) LE () TY () SI	NGTH PE: RANE	IARAC I: 38 nucl EDNE	25 k eic SS:	ase acid doub	pai:	rs							
		(ii)	MOL	ECUL	E TY	PE:	DNA	(ger	nomic	=)							
50		(ix)	FEA (A) NA	ME/K	EY:	CDS	8822									
55		(xi)	SEQ						SEQ I	D NO	0:70	:					
,5	ATG Met	CCA Pro	GTT Val	GCA Ala	ATA Ile 5	AAT Asn	AGT Ser	TTT Phe	AAT Asn	TAT Tyr 10	AAT Asn	GAC Asp	CCT Pro	GTT Val	AAT Asn 15	GAT Asp	48
b()	GAT Asp	ACA Thr	ATT Ile	TTA Leu 20	TAC Tyr	ATG Met	CAG Gln	ATA Ile	CCA Pro 25	TAT Tyr	GAA Glu	GAA Glu	AAA Lys	AGT Ser 30	AAA	AAA Lys	96
5	TAT Tyr	TAT Tyr	AAA Lys 35	GCT Ala	TTT Phe	GAG Glu	ATT Ile	ATG Met 40	CGT Arg	AAT Asn	GTT Val	TGG Trp	ATA Ile 45	ATT Ile	CCT Pro	GAG Glu	144
7()	AGA Arg	AAT Asn 50	ACA Thr	ATA Ile	GGA Gly	ACG Thr	AAT Asn	CCT Pro	AGT Ser	GAT Asp	TTT Phe	GAT Asp	CCA Pro	CCG Pro	GCT Ala	TCA Ser	192

,	TTA Leu 65	AAC Lys	AAC Asr	GGA Gly	AGC Ser	AGT Ser 70	AT a	TAT	TAT	GAT Asp	CCT Pro	Ası	r TA' a Ty:	r TT/	A ACC	C ACT Thr 80		240
. 5	•			-70	85	n. g	171	Dec	ггус	90	Thi	: I16	E Lys	. Let	Phe 95	-		288
10	_			100			nia	GIY	105	vaı	. Leu	Lei	ı Glr	1 Glu	l Il∈	TCA Ser		336
15	•		115		-7-	Deu	Gry	120	Asp	nıs	ınr	Pro	125	Asp	Glu	TTC Phe		384
20		130					135	361	val	ASI	116	140	Leu	Ser	Thr	AAT Asn		432
35	145					150	Deu	ASII	reu	ren	155	Leu	Gly	Ala	Gly	CCT Pro 160		480
25	•				165	CyS	Cys	1 7 1	PIG	170	Arg	Lys	Leu	Ile	Asp 175			528
30			·uı	180	Asp	CCA Pro	ser	ASN	185	Gly	Phe	Gly	Ser	Ile 190	Asn	Ile		576
35			195	5 01	110	GAG Glu	1 7 1	200	Tyr	Tnr	Phe	Asn	Asp 205	Ile	Ser	Gly		624
40	•	210				ACA Thr	215	set	Pne	ıте	Ala	220	Pro	Ala	Ile	Ser		672
45	225			O14	beu .	230	nis	АТА	Leu	HIS	Gly 235	Leu	Tyr	Gly	Ala	Arg 240		720
45	GGA Gly		••••	171	245	GIU	1111	116	GIU	250	Lys	Gln	Ala	Pro	Leu 255	Met		768
50	ATA Ile		Jiu	260	710	rie	Arg	reu	265	GIu	Phe	Leu	Thr	Phe 270	Gly	Gly	1	816
55	CAG Gln		275			110	1112	280	Ald	мес	ràs	Glu	Lys 285	Ile	Tyr	Asn	1	864
60		290	Dea	AIG	NS!!	lyr	295	гàг	пе	Ala	Thr	Arg 300	Leu	Ser	Glu	Val	!	912
, -	AAT Asn 305	Jer	nia	PIO	PIO	310	lyr	Asp	11e	Asn	Glu 315	Tyr	Lys	Asp	Tyr	Phe 320	9	960
65	CAA Gln	rrp	БуБ	Tyr	325	Leu ,	Asp	Lys	Asn	Ala 330	Asp	Gly	Ser	туг	Thr 335	Val	10	800
70	AAT (Asn (GAA Glu	AAT Asn	AAA Lys	TTT . Phe .	AAT (Asn (GAA . Glu	ATT Ile	TAT Tyr	AAA Lys	AAA Lys	TTA Leu	TAT Tyr	AGT Ser	TTT Phe	ACA Thr	10	056

,				340					345					350				
5	GAG Glu	AGT Ser	GAC Asp 355	TTA Leu	GCA Ala	AAT Asn	AAA Lys	TTT Phe 360	AAA Lys	GTA Val	AAA Lys	TGT Cys	AGA Arg 365	AAT Asn	ACT Thr	TAT Tyr	:	1104
10	TTT Phe	ATT Ile 370	AAA Lys	TAT Tyr	GAA Glu	TTT Phe	TTA Leu 375	AAA Lys	GTT Val	CCA Pro	AAT Asn	TTG Leu 380	TTA Leu	GAT Asp	GAT Asp	GAT Asp	:	1152
10	ATT 11e 385	TAT Tyr	ACT Thr	GTA Val	TCA Ser	GAG Glu 390	GGG Gly	TTT Phe	AAT Asn	ATA Ile	GGT Gly 395	AAT Asn	TTA Leu	GCA Ala	GTA Val	AAC Asn 400	3	1200
15	AAT Asn	CGC Arg	GGA Gly	Gln	AGT Ser 405	ATA Ile	AAG Lys	TTA Leu	AAT Asn	CCT Pro 410	AAA Lys	ATT Ile	ATT Ile	GAT Asp	TCC Ser 415	ATT Ile	1	1248
20	CCA Pro	GAT Asp	AAA Lys	GGT Gly 420	CTA Leu	GTA Val	GAA Glu	AAG Lys	ATC Ile 425	GTT Val	AAA Lys	TTT Phe	TGT Cys	AAG Lys 430	AGC Ser	GTT Val	1	1296
25	ATT Ile	CCT Pro	AGA Arg 435	AAA Lys	GGT Gly	ACA Thr	AAG Lys	GCG Ala 440	CCA Pro	CCG Pro	CGA Arg	CTA Leu	TGC Cys 445	ATT Ile	AGA Arg	GTA Val	1	1344
30	AAT Asn	AAT Asn 450	AGT Ser	GAG Glu	TTA Leu	TTT Phe	TTT Phe 455	G TA Val	GCT Ala	TCA Ser	GAA Glu	AGT Ser 460	AGC Ser	TAT Tyr	AAT Asn	GAA Glu	1	1392
	AAT Asn 465	GAT Asp	ATT Ile	AAT Asn	ACA Thr	CCT Pro 470	AAA Lys	GAA Glu	ATT Ile	GAC Asp	GAT Asp 475	ACA Thr	ACA Thr	AAT Asn	CTA Leu	AAT Asn 480	3	1440
35	AAT Asn	AAT Asn	TAT Tyr	AGA Arg	AAT Asn 485	AAT Asn	TTA Leu	GAT Asp	GAA Glu	GTT Val 490	ATT Ile	TTA Leu	GAT Asp	TAT Tyr	AAT Asn 495	AGT Ser	1	1488
40	CAG Gln	ACA Thr	ATA Ile	CCT Pro 500	CAA Gln	ATA Ile	TCA Ser	AAT Asn	CGA Arg 505	ACA Thr	TTA Leu	AAT Asn	ACA Thr	CTT Leu 510	GTA Val	CAA Gln	1	536
45	GAC Asp	AAT Asn	AGT Ser 515	TAT Tyr	GTG Val	CCA Pro	AGA Arg	TAT Tyr 520	GAT Asp	TCT Ser	AAT Asn	GGA Gly	ACA Thr 525	AGT Ser	GAA Glu	ATA Ile	1	1584
50	GAG Glu	GAA Glu 530	TAT Tyr	GAT Asp	GTT Val	GTT Val	GAC Asp 535	TTT Phe	AAT Asn	GTA Val	TTT Phe	TTC Phe 540	TAT Tyr	TTA Leu	CAT His	GCA Ala	1	1632
	CAA Gln 545	AAA Lys	GTG Val	CCA Pro	GAA Glu	GGT Gly 550	GAA Glu	ACC Thr	AAT Asn	ATA Ile	AGT Ser 555	TTA Leu	ACT Thr	TCT Ser	TCA Ser	ATT Ile 560	1	.680
55	GAT Asp	ACA Thr	GCA Ala	TTA Leu	TTA Leu 565	GAA Glu	GAA Glu	TCC Ser	AAA Lys	GAT Asp 570	ATA Ile	TTT Phe	TTT Phe	TCT Ser	TCA Ser 575	GAG Glu	1	.728
60	TTT Phe	ATC Ile	GAT Asp	ACT Thr 580	ATC Ile	AAT Asn	AAA Lys	CCT Pro	GTA Val 585	TAA NSA	GCA Ala	GCA Ala	CTA Leu	TTT Phe 590	ATA Ile	GAT Asp	1	.776
65	TGG Trp	ATA Ile	AGC Ser 595	AAA Lys	GTA Val	ATA Ile	AGA Arg	GAT Asp 600	TTT Phe	ACC Thr	ACT Thr	GAA Glu	GCT Ala 605	ACA Thr	CAA Gln	AAA Lys	1	.824
70	AGT Ser	ACT Thr 610	GTT Val	GAT Asp	AAG Lys	ATT Ile	GCA Ala 615	GAC Asp	ATA Ile	TCT Ser	TTA Leu	ATT Ile 620	GTA Val	CCC Pro	TAT Tyr	GTA Val	1	.872

	GG1 G1 y 625	CT Let	r GC:	TTC Lev	AAT Asn	ATA		ATT	r GAC ≥ Glu	GC/	A GAA A Glu 635	і Гу:	A GGA S Gly	AA? / As:	r TT:	r GAG ≘ Glu 640	1920
5	GAG Glu	GCA Ala	TTI Phe	GAA Glu	TTA Leu 645		GGA Gly	GTC Val	G GGT Gly	AT7 11e	: rea	A TT	A GAA 1 Glu	TTT Phe	GT0 Val	G CCA	1968
10	GAA Glu	CTI Leu	ACA Thr	ATT Ile 660		GTA Val	ATT Ile	TTA Leu	GTG Val 665	Pue	ACG Thr	ATA	AAA Lys	TCC Ser 670	Tyr	ATA Ile	2016
15	•		675			2,3	ASII	680	ATA	ile	Lys	Ala	11e 685	Asn	Asn	TCA Ser	2064
20		690		5		*****	695	тър	Lys	Glu	lle	700	Ser	Trp	Ile		2112
	705					710	110	us!!	1111	GIN	715	Asn	Lys	Arg	Lys	GAG Glu 720	2160
25		-	-,-		725	Deu	OI!!	WPII	GIII	730	Asp	Ala	ATA Ile	Lys	Thr 735	Ala	2208
30			-7-	740	- / -	AJII	ASII	TYL	745	ser	Asp	Glu	AAA Lys	Asn 750	Arg	Leu	2256
35			755		ASII	116	ASII	760	iie	GIu	Glu	Glu	TTG Leu 765	Asn	Lys	Lys	2304
40		770	Dea	AIG	MEC	Lys	775	TIE	Glu	Arg	Phe	Met 780	ACA Thr	Glu	Ser	Ser	2352
	785		- , -	Deu	Mec	790	Leu	116	Asn	GIu	795	Lys	GTT Val	Gly	Lys	800	2400
45	-,-	-,0	. , .	vsb	805	nis	vai	Lys	ser	810	Leu	Leu	AAC Asn	Tyr	Ile 815	Leu	2448
50	, iob		~ • •	820	116	Leu	GIY	GIU	825	Thr	Asn	Glu		Ser 830	Asp	Leu	2496
55			835		beu	ASII	sei	840	116	Pro	Phe	Glu	CTT Leu 845	Ser	Ser	Tyr	2544
60		850	,,,p	шуз	116	rea	855	116	Tyr	Phe	Asn	Arg 860	Leu	Tyr	Lys	Lys	2592
	Ile 865	AAA Lys	GAT Asp	AGT Ser	Ser	ATT Ile 870	TTA (Leu .	GAT Asp	ATG Met	Arg	TAT Tyr 875	GAA Glu	TAA naA	AAT Asn	AAA Lys	TTT Phe 880	2640

	ATA Ile	GAT Asp	ATC Ile	TCT Ser	GGA Gly 885	TAT Tyr	GGT Gly	TCA Ser	AAT Asn	ATA Ile 890	Ser	ATT Ile	' AAT : Asn	GGA Gly	AAC Asn 895	GTA Val	2688
5	TAT Tyr	ATT	TAT Tyr	TCA Ser 900	Thr	AAT Asn	AGA Arg	AAT Asn	CAA Gln 905	Phe	GGA Gly	ATA Ile	TAT	AAT Asn 910	AGT Ser	AGG Arg	2736
10	CTT Leu	AGT Ser	GAA Glu 915	GTT Val	AAT Asn	ATA Ile	GCT Ala	CAA Gln 920	AAT Asn	AAT Asn	GAT Asp	ATT Ile	ATA Ile 925	TAC Tyr	AAT Asn	AGT Ser	2784
15	AGA Arg	TAT Tyr 930	CAA Gln	AAT Asn	TTT Phe	AGT Ser	ATT Ile 935	AGT Ser	TTC Phe	TGG Trp	GTA Val	AGG Arg 940	ATT Ile	CCT Pro	AAA Lys	CAC His	2832
20	945	Lys	Pro	Met	AAT Asn	950	Asn	Arg	Glu	Tyr	Thr 955	Ile	Ile	Asn	Суѕ	Met 960	2880
	GIY	Asn	Asn	Asn	TCG Ser 965	Gly	Trp	Lys	Ile	Ser 970	Leu	Arg	Thr	Val	Arg 975	Asp	2928
25	CÁR	GIU	11e	980 11e	TGG Trp	Thr	Leu	Gln	Asp 985	Thr	Ser	Gly	Asn	Lys 990	Glu	Asn	2976
30	reu	iie	995	Arg	TAT Tyr	Glu	Glu	Leu 1000	Asn)	Arg	Ile	Ser	Asn 1009	Tyr	Ile	Asn	3024
35	rys.	1010	ile	Pne	GTA Val	Thr	Ile 1015	Thr	Asn	Asn	Arg	Leu 1020	Gly	Asn	Ser	Arg	3072
40	1025	lyr	116	Asn	GGA Gly	Asn 1030	Leu	Ile	Val	Glu	Lys 1035	Ser	Ile	Ser	Asn	Leu 1040	3120
	GIY	Asp	IIE	His	GTT Val 1045	Ser	Asp	Asn	Ile	Leu 1050	Phe	Lys	Ile	Val	Gly 1055	Суѕ	3168
45	ASP	ASP	Giu	1060)	Val	Gly	Ile	Arg 1065	Tyr	Phe	Lys	Val	Phe 1070	λsn	Thr	3216
50	GAA Glu	Leu	Asp 1075	Lys	Thr	GIu	Ile	Glu 1080	Thr	Leu	Tyr	Ser	Asn 1085	Glu	Pro	Asp	3264
55		Ser 1090	116	Leu	Lys	Asn	Tyr 1095	Trp	Gly	Asn	Tyr	Leu 1100	Leu	Tyr	Asn	Lys	3312
60	AAA Lys 1105	Tyr	Tyr	Leu	Phe	Asn 1110	Leu	Leu	Arg	Lys	Asp 1115	Lys	Tyr	Ile	Thr	Leu 1120	3360
	AAT Asn	TCA Ser	GGC Gly	ATT Ile	TTA . Leu . 1125	AAT Asn	ATT . Ile .	AAT Asn	CAA Gln	CAA Gln 1130	Arg	GGT Gly	GTT Val	Thr	GAA Glu 1135	Gly	3408
65	TCT Ser	GTT Val	Pue	TTG Leu 1140	Asn '	TAT . Tyr	AAA Lys	Leu	TAT Tyr 1145	Glu	GGA Gly	GTA Val	Glu	GTC . Val 1150	ATT Ile	ATA Ile	3456
70	AGA . Arg :	AAA . Lys .	AAT Asn	GGT Gly	CCT . Pro	ATA (GAT . Asp	ATA Ile	TCT Ser	AAT Asn	ACA Thr	GAT Asp	AAT Asn	TTT (Phe	GTT Val	AGA Arg	3504

	•		115	55				116	0				116	5			
5	AAA Lys	AAC Asn 117	GAT Asp	CTA Leu	GCA Ala	TAC Tyr	ATT Ile 117	ASI	GTA Val	GTA Val	GAT Asp	CGT Arg	Gly	GTA Val	GAZ Glu	TAT Tyr	3552
10	CGG Arg 118		TAT	GCT Ala	GAT Asp	ACA Thr	PAR	TCA Ser	GAG Glu	AAA Lys	GAG Glu 119	Lys	ATA Ile	ATA Ile	AGA Arg	ACA Thr 1200	3600
	TCT Ser	AAT Asn	CTA Leu	AAC Asn	GAT Asp 120	Ser	TTA Leu	GGT Gly	CAA Gln	ATT Ile 121	Ile	GTT Val	ATG Met	GAT Asp	TCA Ser 121	ATA Ile 5	3648
15	GGA Gly	AAT Asn	AAT Asn	TGC Cys 122	TIIL	ATG Met	AAT Asn	TTT Phe	CAA Gln 122	Asn	AAT Asn	AAT Asn	GGG Gly	AGC Ser 123	Asn	ATA Ile	3696
20	GGA Gly	TTA Leu	CTA Leu 123	O T y	TTT Phe	CAT His	TCA Ser	AAT Asn 124	Asn	TTG Leu	GTT Val	GCT Ala	AGT Ser 124	Ser	TGG Trp	TAT Tyr	3744
25	TAT Tyr	AAC Asn 125		ATA Ile	CGA Arg	AGA Arg	AAT Asn 1255	TILL	AGC Ser	AGT Ser	AAT Asn	GGA Gly 126	Cys	TTT Phe	TGG Trp	AGT Ser	3792
30	TCT Ser 1265	TTG	TCT Ser	AAA Lys	GAG Glu	AAT Asn 1270	Gly	TGG Trp	AAA Lys	GAA Glu	TGA						3825
	(2)					SEQ											
35			(i) S	(A) (B)	LEI	CHAR IGTH: PE: a	127 mino	4 an	nino d	acio	is					·	
		()	ii) N	OLE	CULE	TYPE	: pr	otei	.n								
40						DESC											
	Met 1	Pro	Val	Ala	Ile 5	Asn	Ser	Phe	Asn	Tyr 10	Asn	Asp	Pro	Val	Asn 15	Asp	
45	Asp			20					25					3 C			
50	Tyr '	Tyr	Lys 35	Ala	Phe	Glu	Ile	Met 40	Arg	Asn	Val	Trp	Ile 45	Ile	Pro	Glu	
	Arg /						22					60					
55	Leu 1	Lys	Asn	Gly	Ser	Ser 70	Ala	Tyr	Tyr	Asp	Pro 75	Asn	Tyr	Leu	Thr	Thr 80	
	Asp /	Ala	Glu	Lys	Asp 85	Arg	Tyr	Leu	Lys	Thr 90	Thr	Ile	Lys	Leu	Phe 95	Lys	
60	Arg :			100					105					110			
65	Tyr 1	Ala	Lys 115	Pro	Tyr	Leu	Gly .	Asn 120	Asp	His	Thr	Pro	Ile 125	Asp	Glu	Phe	
-							135					140					
70	Val (Slu	Ser	Ser	Met	Leu 150	Leu /	Asn	Leu	Leu	Val 155	Leu	Gly	Ala	Gly	Pro 160	

ı	Asp) Ile	e Phe	e Gl	Ser 165	Cys	cy:	s Tyr	Pro	Va]	Arg	Lys	Leu	ı Ile	Asp 175	
5	Asp	Val	l Val	180	r Asp	Pro	Ser	Asn	185	Gly	/ Phe	Gly	' Ser	11e		Ile
	Val	Thi	Phe 195	e Sei	Pro	Glu	Туг	Glu 200	Tyr	Thr	Phe	Asn	Asp 205		Ser	Gly
10	Gly	His 210	s Asr	ı Ser	Ser	Thr	Glu 215	ser	Phe	Ile	Ala	Asp 220	Pro	Ala	Ile	Ser
15	Leu 225	Ala	His	Glu	Leu	Ile 230	His	Ala	Leu	His	Gly 235	Leu	Tyr	Gly	Ala	Arg 240
	Gly	Val	Thr	туг	Glu 245	Glu	Thr	Ile	Glu	Val 250	Lys	Gln	Ala	Pro	Leu 255	Met
20	Ile	Ala	Glu	Lys 260	Pro	Ile	Arg	Leu	Glu 265	Glu	Phe	Leu	Thr	Phe 270	Gly	Gly
	Gln	Asp	Leu 275	Asn	Ile	Ile	Thr	Ser 280	Ala	Met	Lys	Glu	Lys 285	Ile	Tyr	Asn
25	Asn	Leu 290	Leu	Ala	Asn	Tyr	Glu 295	Lys	Ile	Ala	Thr	Arg 300	Leu	Ser	Glu	Val
30	Asn 305	Ser	Ala	Pro	Pro	Glu 310	Tyr	Asp	Ile	Asn	Glu 315	Tyr	Lys	Asp	Tyr	Phe 320
	Gln	Trp	Lys	Tyr	Gly 325	Leu	Asp	Lys	Asn	Ala 330	Asp	Gly	Ser	туг	Thr 335	Val
35	Asn	Glu	Asn	Lys 340	Phe	Asn	Glu	Ile	Tyr 345	Lys	Lys	Leu	Tyr	Ser 350	Phe	Thr
	Glu	Ser	Asp 355	Leu	Ala	Asn	Lys	Phe 360	Lys	Val	Lys	Cys	Arg 365	Asn	Thr	Tyr
4()	Phe	Ile 370	Lys	Tyr	Glu	Phe	Leu 375	Lys	Val	Pro	Asn	Leu 380	Leu	Asp	Asp	Asp
45	Ile 385	Tyr	Thr	Val	Ser	Glu 390	Gly	Phe	Asn	Ile	Gly 395	Asn	Leu	Ala		Asn 400
	Asn	Arg	Gly	Gln	Ser 405	Ile	Lys	Leu	Asn	Pro 410	Lys	Ile	Ile	Asp	Ser 415	Ile
50	Pro	Asp	Lys	Gly 420	Leu	Val	Glu	Lys	11e 425	Val	Lys	Phe	Cys	Lys 430	Ser	Val
	Ile	Pro	Arg 435	Lys	Gly	Thr	Lys	Ala 440	Pro	Pro	Arg	Leu	Cys 445	Ile	Arg	Val
55	Asn	Asn 450	Ser	Glu	Leu	Phe	Phe 455	Val	Ala	Ser	Glu	Ser 460	Ser	Tyr	Asn	Glu
50	Asn 465	Asp	Ile	Asn	Thr	Pro 470	Lys	Glu	Ile	Asp	Asp 475	Thr	Thr	Asn		Asn 480
	Asn	Asn	Tyr	Arg	Asn 485	Asn	Leu	Asp	Glu	Val 490	Ile	Leu	Asp	Tyr	Asn :	Ser
55	Gln	Thr	lle	Pro 500	Gln	Ile	Ser	Λsn	A rg 5 05	Thr	Leu	Asn	Thr	Leu 510	Val (Gln
	Asp	Asn	Ser 515	Tyr	Val	Pro	Arg	Tyr 520	Asp	Ser	Asn		Thr 525	Ser	Glu :	lle
0'	Glu	Glu	Tyr	Asp	Val	Val	Asp	Phe	Asn	Val	Phe	Phe	туг	Leu	His A	Λla

		530					535					540				
5	Gln 545	Lys	Val	Pro	Glu	Gly 550	Glu	Thr	Asn	Ile	Ser 555	Leu	Thr	Ser	Ser	Ile 560
					505					570					57 5	
10				300					585					590		Asp
15								600					605			Lys
15							013					620				Val
20						030					635					Glu 640
					043					Ile 650					655	
25				500					665	Phe				670		
30			07.5					680		Ile			685			
30							095			Glu		700				
35						/10				Gln	715			•		720
					723					Val 730					735	
40				740					745	Ser				750		
45			,,,					760		Glu			765			
••		,,,					//5			Arg		780				
50	.05					790				Glu	795					800
					803					Asp 810					815	
55				620					825	Thr				830		
60			033					840		Pro			845			
.,0		030					855			Phe		860				
65	503					870					875					880
					003					Ile 890					895	
70	ıyr	116	ıyr	Ser 900	Thr	Asn	Arg	Asn	Gln 905	Phe	Gly	Ile	Tyr	Asn 910	Ser	Arg

1	Leu	Se1	915	ı Val	Asn	Ile	Ala	Gln 920	Asr	n Asn	Asp	Ile	11e 925	Tyr	Asn	Ser
5	Arg	930	Glr	a Asr	Phe	Ser	11e 935	Ser	Phe	Trp	Val	Arg 940		Pro	Lys	His
	Tyr 945	Lys	Pro	Met	Asn	His 950	Asn	Arg	Glu	Tyr	Thr 955	Ile	Ile	Asn	Cys	Met 960
10	Gly	Asn	Asn	Asn	Ser 965	Gly	Trp	Lys	Ile	Ser 970	Leu	Arg	Thr	Val	Arg 975	
15	Cys	Glu	Ile	980	Trp	Thr	Leu	Gln	Asp 985	Thr	Ser	Gly	Asn	Lys 990		Asn
	Leu	Ile	995	Arg	Туг	Glu	Glu	Leu 100	Asn 0	Arg	Ile	Ser	Asn 100	Tyr 5	Ile	Asn
20	Lys	Trp 101	lle 0	Phe	Val	Thr	Ile 101	Thr 5	Asn	Asn	Arg	Leu 102	Gly 0	Asn	Ser	Arg
	Ile 102	Tyr 5	Ile	Asn	Gly	Asn 103	Leu O	Ile	Val	Glu	Lys 103	Ser 5	Ile	Ser	Asn	Leu 104
25	Gly	Asp	Ile	His	Val 104	Ser 5	Asp	Asn	Ile	Leu 1050	Phe	Lys	Ile	Val	Gly 105	
30	Asp	Asp	Glu	Thr 106	Tyr 0	Val	Gly	Ile	Arg 106	Tyr 5	Phe	Lys	Val	Phe 107		Thr
	Glu	Leu	Asp 107	Lys 5	Thr	Glu	Ile	Glu 108	Thr	Leu	Tyr	Ser	Asn 1089		Pro	Asp
35	Pro	Ser 109	Ile O	Leu	Lys	Asn	Tyr 1099	Trp 5	Gly	Asn	Tyr	Leu 1100		Tyr	Asn	Lys
	Lys 1109	Tyr 5	Tyr	Leu	Phe	Asn 1110	Leu)	Leu	Λrg	Lys	Asp 1119	Lys 5	Tyr	lle	Thr	Leu 1120
4()	Asn	Ser	Gly	Ile	Leu 1129	Asn	Ile	Asn	Gln	Gln 1130		Gly	Val	Thr	Glu 1139	
45	Ser	Val	Phe	Leu 1140	Asn O	Tyr	Lys	Leu	Tyr 1149	Glu 5	Gly	Val	Glu	Val 1150		Ile
ı	Arg	Lys	Asn 1159	Gly 5	Pro	Ile	Asp	Ile 1160	Ser	Asn	Thr	Дsр	Asn 1165		Val	Arg
50		11/0	J				1179	5		Val		1180				-
	110	•				1190	l			Lys	1195	i				1200
55	Ser	Asn	Leu	Asn	Asp 1205	Ser	Leu	Gly	Gln	Ile 1210	Ile	Val	Met	Asp	Ser 1215	
5()	Gly	Asn	Asn	Cys 1220	Thr	Met	Asn	Phe	Gln 1225	Asn	Asn	Asn	Gly	Ser 1230		Ile
	Gly	Leu	Lėu 1235	Gly	Phe	His	Ser	Asn 1240	Asn	Leu	Val		Ser 1245		Trp	Tyr
5		1250	,				1255			Ser	Asn	Gly 1260		Phe	Trp	Ser
	Ser 1265	lle	Ser	Lys	Glu	As n 1270	Gly	Trp	Lys	Glu						
'()	(2)	TNEC	דגשם	TON	EOD	CEO	TD									

5		(:		EQUEN (A) I (B) I (C) S (D) I	LENG: TYPE: STRAI	TH: : : nuc NDEDN	1460 Cleid NESS:	base ac: do:	e pa: id	irs							
		(ii	L) MO	LEC	JLE 1	YPE:	DNA	k (ge	nomi	(c)							
10		(i)	(ATUR (A) N (B) L	IAME/	KEY:	CDS	; 14	151								
		(xi) SE	QUEN	CE D	ESCR	IPTI	ON:	SEQ	ID N	10:72	! :					
15	AGA	TCTC	GAT	CCCG	CGAA	T TA	'AATA	CGAC	T CA	CTAT	'AGGG	GAA	TTGT	GAG	CGGA	TAACAA	60
														ATG	GGC	CAT	116
20														1			
	CAT His	CAT His 5		CAT His	CAT His	CAT His	CAT His	HIS	CAC His	AGC Ser	AGC Ser	GGC Gly	His	ATC Ile	GAA Glu	GGT Gly	164
25	CGT Arg 20	CAT His	ATG Met	GCT Ala	AGC Ser	ATG Met 25	ALA	ATT Ile	CTA Leu	ATT Ile	ATA Ile 30	Tyr	TTT Phe	TAA Asn	AGA Arg	TTA Leu 35	212
30	TAT Tyr	lys Lys	AAA Lys	ATT Ile	AAA Lys 40	ASP	AGT Ser	TCT Ser	ATT	TTA Leu 45	GAT Asp	ATG Met	CGA Arg	TAT Tyr	GAA Glu 50	AAT Asn	260
35	AAT Asn	AAA Lys	TTT Phe	ATA Ile 55	GAT Asp	ATC Ile	TCT Ser	GGA Gly	TAT Tyr 60	GGT Gly	TCA Ser	AAT Asn	ATA Ile	AGC Ser 65	Ile	AAT Asn	308
40	GGA Gly	'AAC Asn	GTA Val 70	TAT Tyr	ATT Ile	TAT Tyr	TCA Ser	ACA Thr 75	AAT Asn	AGA Arg	AAT Asn	CAA Gln	TTT Phe 80	GGA Gly	ATA Ile	TAT Tyr	356
	AAT Asn	AGT Ser 85	AGG Arg	CTT Leu	AGT Ser	GAA Glu	GTT Val 90	AAT Asn	ATA Ile	GCT Ala	CAA Gln	AAT Asn 95	AAT Asn	GAT Asp	ATT Ile	ATA Ile	404
45	TAC Tyr 100	AAT Asn	AGT Ser	AGA Arg	TAT Tyr	CAA Gln 105	AAT Asn	TTT Phe	AGT Ser	ATT Ile	AGT Ser 110	TTC Phe	TGG Trp	GTA Val	AGG Arg	ATT Ile 115	452
50	CCT Pro	AAA Lys	CAC H1s	TAC Tyr	AAA Lys 120	CCT Pro	ATG Met	AAT Asn	CAT His	AAT Asn 125	CGG Arg	GAA Glu	TAC Tyr	ACT Thr	ATA Ile 130	ATA Ile	500
55	AAT Asn	TGT Cys	ATG Met	GGG Gly 135	AAT Asn	AAT Asn	AAT Asn	TCG Ser	GGA Gly 140	TGG Trp	AAA Lys	ATA Ile	TCA Ser	CTT Leu 145	AGA Arg	ACT Thr	548
60	GTT Val	AGA Arg	GAT Asp 150	TGT Cys	GAA Glu	ATA Ile	ATT Ile	TGG Trp 155	ACT Thr	TTA Leu	CAA Gln	GAT Asp	ACT Thr 160	TCT Ser	GGA Gly	AAT Asn	596
	AAG Lys	GAA Glu 165	AAT Asn	TTA Leu	ATT Ile	TTT Phe	AGG Arg 170	TAT Tyr	GAA Glu	GAA Glu	CTT Leu	AAT Asn 175	AGG Arg	ATA Ile	TCT Ser	AAT Asn	644
65	TAT Tyr 180	ATA Ile	AAT Asn	AAA Lys	TGG Trp	ATT Ile 185	TTT Phe	GTA Val	ACT Thr	ATT Ile	ACT Thr 190	AAT Asn	AAT Asn	AGA Arg	TTA Leu	GGC Gly 195	692
70	AAT Asn	TCT Ser	AGA Arg	ATT Ile	TAC Tyr	ATC Ile	AAT Asn	GGA Gly	AAT Asn	TTA Leu	ATA Ile	GTT Val	GAA Glu	AAA Lys	TCA Ser	ATT Ile	740

	-				200					205					210		
5	TCG Ser	AAT Asn	TTA Leu	GGT Gly 215	Asp	ATT	CAT His	GTI Val	Ser 220	Asp	AAT Asn	ATA	TTA Leu	TTT Phe 225	Lys	ATT Ile	788
10	GTT Val	GGT Gly	TGT Cys 230	Asp	GAT Asp	GAA Glu	ACG Thr	TAT Tyr 235	Val	GGT Gly	ATA Ile	AGA Arg	TAT Tyr 240	TTT Phe	AAA Lys	GTT Val	836
	TTT Phe	AAT Asn 245	ACG Thr	GAA Glu	TTA Leu	GAT Asp	AAA Lys 250	Thr	GAA Glu	ATT Ile	GAG Glu	ACT Thr 255	TTA Leu	TAT Tyr	AGT Ser	AAT Asn	884
15	GAG Glu 260	PIO	GAT Asp	CCA	AGT Ser	ATC Ile 265	TTA Leu	AAA Lys	AAC Asn	TAT Tyr	TGG Trp 270	GGA Gly	AAT Asn	TAT Tyr	TTG Leu	CTA Leu 275	932
20	TAT Tyr	TAA Asn	AAA Lys	AAA Lys	TAT Tyr 280	TAT Tyr	TTA Leu	TTC Phe	AAT Asn	TTA Leu 285	CTA Leu	AGA Arg	AAA Lys	GAT Asp	AAG Lys 290	TAT Tyr	980
25	ATT Ile	ACT Thr	CTG Leu	AAT Asn 295	TCA Ser	GGC Gly	ATT Ile	TTA Leu	AAT Asn 300	ATT Ile	AAT Asn	CAA Gln	CAA Gln	AGA Arg 305	GGT Gly	GTT Val	1028
30	ACT Thr	GAA Glu	GGC Gly 310	TCT Ser	GTT Val	TTT Phe	TTG Leu	AAC Asn 315	TAT Tyr	AAA Lys	TTA Leu	TAT Tyr	GAA Glu 320	GGA Gly	GTA Val	GAA Glu	1076
	GTC Val	ATT Ile 325	ATA Ile	λGA Arg	AAA Lys	AAT Asn	GGT Gly 330	CCT Pro	ATA Ile	GAT Asp	ATA Ile	TCT Ser 335	AAT Asn	ACA Thr	GAT Asp	AAT Asn	1124
35	TTT Pḥe 340	GTT Val	AGA Arg	AAA Lys	AAC Asn	GAT Asp 345	CTA Leu	GCA Ala	TAC Tyr	ATT Ile	AAT Asn 350	GTA Val	GTA Val	GAT Asp	CGT Arg	GGT Gly 355	1172
40	GTA Val	GAA Glu	TAT Tyr	CGG Arg	TTA Leu 360	TAT Tyr	GCT Ala	GAT Asp	ACA Thr	AAA Lys 365	TCA Ser	GAG Glu	AAA Lys	GAG Glu	AAA Lys 370	ATA Ile	1220
45	ATA Ile	AGA Arg	ACA Thr	TCT Ser 375	AAT Asn	CTA Leu	AAC Asn	GAT Asp	AGC Ser 380	TTA Leu	GGT Gly	CAA Gln	ATT Ile	ATA Ile 385	GT T Val	ATG Met	1268
50	GA T Asp	TCA Ser	ATA Ile 390	GGA Gly	AAT Asn	AAT Asn	TGC Cys	ACA Thr 395	ATG Met	AAT Asn	TTT Phe	CAA Gln	AAC Asn 400	AAT Asn	AAT Asn	GGG Gly	1316
	AGC Ser	AAT Asn 405	ATA Ile	GGA Gly	TTA Leu	CTA Leu	GGT Gly 410	TTT Phe	CAT His	TCA Ser	AAT Asn	AAT Asn 415	TTG Leu	GTT Val	GCT Ala	AGT Ser	1364
55	AGT Ser 420	TGG Trp	TAT Tyr	TAT Tyr	AAC Asn	AAT Asn 425	ATA Ile	CGA Arg	AGA Arg	AAT Asn	ACT Thr 430	AGC Ser	AGT .	AAT Asn	Gly	TGC Cys 435	1412
60	TTT Phe	TGG Trp	AGT Ser	ser	ATT Ile 440	TCT Ser	AAA Lys	GAG Glu	Asn	GGA Gly 445	TGG Trp	AAA Lys	GAA Glu	TGAA.	AGCT	T	1460
	(2)	INFO	RMAT	ION	FOR	SEQ	ID N	0:73	:								
65		(i) S	(A) (B)	LEN TYP	CHAR GTH: E: a OLOG	448 mino	ami aci	no a d	cids							
70		(i	i) M														

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

Met Gly His His His His His His His His His Ser Ser Gly His 5 Ile Glu Gly Arg His Met Ala Ser Met Ala Ile Leu Ile Ile Tyr Phe Asn Arg Leu Tyr Lys Lys Ile Lys Asp Ser Ser Ile Leu Asp Met Arg 10 Tyr Glu Asn Asn Lys Phe Ile Asp Ile Ser Gly Tyr Gly Ser Asn Ile 15 Ser Ile Asn Gly Asn Val Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe Gly Ile Tyr Asn Ser Arg Leu Ser Glu Val Asn Ile Ala Gln Asn Asn 20 Asp Ile Ile Tyr Asn Ser Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp 105 Val Arg Ile Pro Lys His Tyr Lys Pro Met Asn His Asn Arg Glu Tyr 25 120 Thr Ile Ile Asn Cys Met Gly Asn Asn Asn Ser Gly Trp Lys Ile Ser 135 30 Leu Arg Thr Val Arg Asp Cys Glu Ile Ile Trp Thr Leu Gln Asp Thr Ser Gly Asn Lys Glu Asn Leu Ile Phe Arg Tyr Glu Glu Leu Asn Arg 35 Ile Ser Asn Tyr Ile Asn Lys Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Gly Asn Ser Arg Ile Tyr Ile Asn Gly Asn Leu Ile Val Glu 40 Lys Ser Ile Ser Asn Leu Gly Asp Ile His Val Ser Asp Asn Ile Leu 215 45 Phe Lys Ile Val Gly Cys Asp Asp Glu Thr Tyr Val Gly Ile Arg Tyr 235 235 240 Phe Lys Val Phe Asn Thr Glu Leu Asp Lys Thr Glu Ile Glu Thr Leu 50 Tyr Ser Asn Glu Pro Asp Pro Ser Ile Leu Lys Asn Tyr Trp Gly Asn 265 Tyr Leu Leu Tyr Asn Lys Lys Tyr Tyr Leu Phe Asn Leu Leu Arg Lys 55 Asp Lys Tyr Ile Thr Leu Asn Ser Gly Ile Leu Asn Ile Asn Gln Gln 60 Arg Gly Val Thr Glu Gly Ser Val Phe Leu Asn Tyr Lys Leu Tyr Glu 310 315 Gly Val Glu Val Ile Ile Arg Lys Asn Gly Pro Ile Asp Ile Ser Asn 65 Thr Asp Asn Phe Val Arg Lys Asn Asp Leu Ala Tyr Ile Asn Val Val Asp Arg Gly Val Glu Tyr Arg Leu Tyr Ala Asp Thr Lys Ser Glu Lys 70

	Glu	Lys 370	Ile	Ile	Arg	Thr	Ser 375		Leu	Asn	Asp	Ser 380	Leu	Gly	Gln	Ile	
5	Ile 385	Val	Met	Asp	Ser	Ile 390	Gly	Asn	Asn	Cys	Thr 395	Met	Asn	Phe	Gln	Asn 400	
	Asn	Asn	Gly	Ser	Asn 405	Ile	Gly	Leu	Leu	Gly 410	Phe	His	Ser	'Asn	Asn 415	Leu	
10	Val	Ala	Ser	Ser 420	Trp	Tyr	Tyr	Asn	Asn 425	Ile	Arg	Arg	Asn	Thr 430	Ser	Ser	
15	Asn	Gly	Cys 435	Phe	Trp	Ser	Ser	Ile 440	Ser	Lys	Glu	Asn	Gly 445	Trp	Lys	Glu	
	(2)	INFO	ORMA:	rion	FOR	SEQ	ID 1	NO : 7	4:								
20		(1)	() () ()	QUENCA) LI B) T' C) S' C) T'	ENGTI YPE : TRANI	i: 33 nucl	3 bas leic ESS:	se pa acio sino	airs 1								
25		(ii)		LECUI													
		(Mi)	SEC	OUEN	CE DE	ESCRI	IPTIC	ON: S	SEQ :	ID NO	0:74	:					
30				ATTCI						3							33
	(2)			rion		-											
35		(1)	(QUENC A) LI B) TY C) ST O) TO	ENGTI PE : PRANI	i: 29 nucl DEDNE	bas leic ESS:	acio sing	airs 1								
40		(ii)		LECUI													
				QUENC					SEQ I	D NO	0:75	:					
45				ATTCI													2.5
ı.	(3)			LION													
50		(1)	(# (E (C	QUENC (A) LE (B) TY (C) ST (C) T(ENGTH PE: PRANE	i: 36 nucl EDNE	394 h Leic ESS:	ase acid doub	pai:	rs							
55		(ii)	MOI	LECUI	LE TY	PE:	DNA	(ger	nomic	=)							
		(ix)	(2	ATURE A) NA B) LO	ME/K			3891									
60		(x1)	SEC	QUENC	CE DE	SCRI	PTIC	N: 9	SEQ 1	D NC):76:						
45	ATG Met	CCA Pro	GTT Val	AAT Asn	ATA Ile 5	AAA Lys	AAC Asn	TTT Phe	AAT Asn	TAT Tyr 10	AAT Asn	GAC Asp	CCT Pro	ATT Ile	AAT Asn 15	AAT Asn	4.8
65	GAT Asp	GAC Asp	ATT Ile	ATT Ile 20	ATG Met	ATG Met	GAA Glu	CCA Pro	TTC Phe 25	AAT Asn	GAC Asp	CCA Pro	GGG Gly	CCA Pro 30	GGA Gly	ACA Thr	96
70	TAT	TAT	AAA	GCT	ттт	AGG	ATT	АТА	GAT	CGT	ATT	TGG	АТА	GTA	CCA	GAA	144

	Tyr	туг	Ly:	s Ala	a Phe	≥ Arg	, Ile	2 Ile 40	e Asp	Ar	g Ile	e Tr	o Il.		l Pro	o Glu	٠	·
5	7.	50	•	-,-	,		55	FIC	ASE) GII	ı Phe	Ası 60	n Ala	a Se	r Th	A GGA r Gly	•	192
01	65			•		70	- 7 -	010	TYL	1 7 1	75) Pro	Thi	туі	r Lei	A AAA Lys 80		240
15		-			85		~, 5	1110	neu	90 90	int	мет	: 116	. Lys	Leu 95			288
		_		100		2,5	710	ser	105	GIN	Arg	Leu	Leu	Asp	Met	ATA		336
20	GTA Val	GAT Asp	GCT Ala 115	ATA Ile	CCT Pro	TAT Tyr	CTT Leu	GGA Gly 120	AAT Asn	GCA Ala	TCT Ser	ACA Thr	CCG Pro	Pro	GAC Asp	AAA Lys		384
25		130					135	Val	ser	116	Asn	Lys 140	Lys	Ile	Ile			432
30	145	-			,,,,,	CAA Gln 150	110	цуз	GIY	Leu	Met 155	Thr	Asn	Leu	Ile	Ile 160		480
35		•		/	165	GTT Val	Dea	ser	ASP	170	Pne	Thr	Asp	Ser	Met 175	Ile		528
			,	180		CCA Pro	116	ser	185	GIY	Pne	Gly	Ala	Arg 190	Met	Met		576
40	ATA Ile	AGA Arg	TTT Phe 195	TGT Cys	CCT Pro	AGT Ser	TGT Cys	TTA Leu 200	AAT Asn	GTA Val	TTT Phe	AAT Asn	AAT Asn 205	GTT Val	CAG Gln	GAA Glu		624
45		210					215	SEI	Arg	Arg	Ala	Tyr 220	Phe	Ala	Asp	Pro		672
50	225		-			CAT His 230	GIU	Leu	iie	HIS	235	Leu	His	Gly	Leu	Tyr 240		720
55	GGA Gly	ATT . Ile	AAG Lys		AGT Ser 245	AAT Asn	TTA Leu	CCA Pro	TTE	ACT Thr 250	CCA Pro	AAT Asn	ACA Thr	AAA Lys	GAA Glu 255	TTT Phe		768
•	TTC .	ATG Met	CAA Gln	CAT His 260	AGC Ser	GAT (CCT Pro	vai	CAA Gln 265	GCA Ala	GAA Glu	GAA Glu	CTA Leu	TAT Tyr 270	ACA Thr	TTC Phe		816
60	GGA (Gly (GGA (Gly)	CAT His 275	GAT Asp	CCT Pro	AGT (Ser)	vaı	ATA Ile 280	AGT Ser	CCT Pro	TCT Ser	ACG Thr	GAT Asp 285	ATG Met	AAT Asn	ATT Ile		864
65	TAT A	AAT A Asn 1 290	AAA Lys	GCG Ala	TTA (Leu (GIII 1	AAT Asn 295	TTT Phe	CAA (Gln ,	GAT Asp	He	GCT Ala 300	AAT Asn	AGG Arg	CTT Leu	AAT Asn		912
70	ATT (Ile V 305	GTT :	ICA . Ser .	AGT (CAA (Gln (310	GGG /	AGT (Ser (G GA A	116	GAT Asp 315	ATT Ile	TCC Ser	TTA Leu	Tyr	AAA Lys 320		960

	CAA Gln	ATA Ile	TAT Tyr	AAA Lys	AAT Asn 325	Lys	TAT Tyr	GAT Asp	TTT Phe	GTT Val 330	Glu	GAT Asp	CCT Pro	AAT Asn	GGA Gly 335	AAA Lys	1	008
5 .	TAT Tyr	AGT Ser	GTA Val	GAT Asp 340	Lys	GAT Asp	AAG Lys	TTT Phe	GAT Asp 345	AAA Lys	TTA Leu	TAT Tyr	AAG Lys	GCC Ala 350	Leu	ATG Met	1	056
10	TTT Phe	GGC Gly	TTT Phe 355	ACT Thr	GAA Glu	ACT Thr	AAT Asn	CTA Leu 360	GCT Ala	GGT Gly	GAA Glu	TAT Tyr	GGA Gly 365	ATA Ile	AAA Lys	ACT Thr	1:	104
15	AGG Arg	TAT Tyr 370	TCT Ser	TAT Tyr	TTT Phe	AGT Ser	GAA Glu 375	TAT Tyr	TTG Leu	CCA Pro	CCG Pro	ATA Ile 380	AAA Lys	ACT	GAA Glu	AAA Lys	1:	152
20	TTG Leu 385	TTA Leu	GAC Asp	AAT Asn	ACA Thr	ATT Ile 390	TAT Tyr	ACT Thr	CAA Gln	AAT Asn	GAA Glu 395	GGC Gly	TTT Phe	AAC Asn	ATA Ile	GCT Ala 400	13	200
	ser	Lys	Asn	Leu	AAA Lys 405	Thr	Glu	Phe	Asn	Gly 410	Gln	Asn	Lys	Ala	Val 415	Asn	12	248
25	rys	GIU	Ala	Tyr 420	GAA Glu	Glu	Ile	Ser	Leu 425	Glu	His	Leu	Val	11e 430	Tyr	Arg	12	296
30	ATA Ile	GCA Ala	ATG Met 435	TGC Cys	AAG Lys	CCT Pro	GTA Val	ATG Met 440	TAC Tyr	AAA Lys	AAT Asn	ACC Thr	GGT Gly 445	AAA Lys	TCT Ser	GAA Glu	13	344
35	Gln	Cys 450	Ile	Ile	GTT Val	Asn	Asn 455	Glu	Asp	Leu	Phe	Phe 460	Ile	Ala	Asn	Lys	13	92
40	465	Ser	Phe	Ser	AAA Lys	470	Leu	Ala	Lys	Ala	Glu 475	Thr	Ile	Ala	Tyr	Asn 480	14	40
	ACA Thr	CAA Gln	AAT Asn	AAT Asn	ACT Thr 485	ATA Ile	GAA Glu	AAT Asn	AAT Asn	TTT Phe 490	TCT Ser	ATA Ile	GAT Asp	CAG Gln	TTG Leu 495	ATT Ile	14	88
45	Leu	Asp	Asn	500	TTA Leu	Ser	Ser	Gly	Ile 505	Asp	Leu	Pro	Asn	Glu 510	Asn	Thr	15	36
50	Glu	Pro	Phe 515	Thr	AAT Asn	Phe	Asp	Asp 520	Ile	Asp	Ile	Pro	Val 525	Tyr	Ile	Lys	15	84
55	Gin	530	Ala	Leu	AAA Lys	Lys	Ile 535	Phe	Val	Asp	Gly	Asp 540	Ser	Leu	Phe	Glu	16	32
60	Tyr 545	Leu	His	Ala	CAA Gln	Thr 550	Phe	Pro	Ser	Asn	11e 555	Glu	Asn	Leu	Gln	Leu 560	16	80
	Thr	Asn	Ser	Leu	AAT Asn 565	qzA	Ala	Leu	Arg	Asn 570	Asn	Asn	Lys	Val	Tyr 575	Thr	17	28
65	TTT Phe	TTT Phe	TCT Ser	ACA Thr 580	AAC Asn	CTT Leu	GTT Val	GAA Glu	AAA Lys 585	GCT Ala	AAT Asn	ACA Thr	GTT Val	GTA Val 590	GGT Gly	GCT Ala	17	76
70	TCA Ser	CTT Leu	TTT Phe	GTA Val	AAC Asn	TGG Trp	GTA Val	AAA Lys	GGA Gly	GTA Val	ATA Ile	GAT Asp	GAT Asp	TTT Phe	ACA Thr	TCT Ser	18	24

	•		59	5				60	0				60	5			
5	GA: Gli	A TC	C AC. r Th	A CAI	A AAJ	A AGT S Ser	ACT Thi		A GAT 2 Asp	T AAA	GTI Val	TC/ Ser 620	A GA' r As _l		A TC	C ATA r Ile	1872
10	625	5		/ -		630		, 410	a Let	AST	635	GI	/ Ası	ı Glı	ı Th:	A GCT r Ala 640	1920
	Lys	GAZ Glu	A AAT 1 Asr	r TTI 1 Phe	AAA Lys 645		GCT Ala	TTT Phe	GAA Glu	ATA Ile 650	GIY	GG# Gly	A GCC	GCT Ala	T ATO	TTA	1968
15	ATG Met	GAC Glu	TTI Phe	ATT lle 660	CCA Pro	GAA Glu	CTT Leu	ATT	GTA Val 665	Pro	ATA Ile	GT1 Val	GG#	TTT Phe 670	TT1	ACA Thr	2016
20			675	1		1		680	GIY	nis	116	116	685	Thr	Ile	TCC Ser	2064
25		690		_,_	-/-	9	695	GIII	БУЗ	irp	Thr	700	Met	Tyr	Gly		2112
30	705					CTC Leu 710	501	1111	Val	ASI	715	GIn	Phe	Tyr	Thr	Ile 720	2160
	AAA Lys	GAA Glu	AGA Arg	ATG Met	TAC Tyr 725	AAT Asn	GCT Ala	TTA Leu	AAT Asn	AAT Asn 730	CAA Gln	TCA Ser	CAA Gln	GCA Ala	ATA Ile 735	GAA Glu	2208
35	AAA Lys	ATA Ile	ATA Ile	GAA Glu 740	GAT Asp	CAA Gln	TAT Tyr	AAT Asn	AGA Arg 745	TAT Tyr	AGT Ser	GAA Glu	GAA Glu	GAT Asp 750		ATG Met	2256
40			755		nop	TTT Phe	ASII	760	iie	Asp	Phe	Lys	Leu 765	Asn	Gln	Ser	2304
45		770				AAC Asn	775	116	Asp	Asp	Phe	780	Asn	Gln	Cys	Ser	2352
50	785	_	- / -			AAT Asn 790	Arg	Met	116	Pro	195	Ala	Val	Lys	Lys	Leu 800	2400
		-			805	AAT Asn	beu	Буѕ	Arg	810	Leu	Leu	Glu	Tyr	Ile 815	Asp	2448
55				820	-,-	TTA Leu	Deu	Asp	825	vai	Asn	lle	Leu	Lys 830	Ser	Lys	2496
60			835		Deu	AAA (-sp	840	116	Pro	Phe .	Asp	Leu 845	Ser	Leu	Tyr	2544
65		850	p		116		355	GIN	vai	Pne .	Asn .	Asn 860	Tyr	Ile	Ser	Asn	2592
70	ATT Ile 865	AGT Ser	AGT Ser	AAT Asn		ATT : Ile I 870	TTA . Leu :	AGT Ser	TTA . Leu :	ser	TAT A	AGA Arg	GGT Gly	GGG Gly	Arg	TTA Leu 880	2640

	ATA Ile	GAT Asp	TC/ Sei	A TC:	F GG/ F Gl ₃ 885	ıyr	GGT Gly	GCA Ala	A ACT	ATC Met	Asr	r GT ı Va	A GG	r TC/ / Se	A GAT C Asp 899	r GTT P Val		2688
5	λTC Ile	TT1 Phe	AAT Asi	GAT Asp 900	, 176	GGA Gly	AAT Asn	GG1 Gly	CAA Glr 905	1 Phe	AAA Lys	TTI Lev	A AA1 1 Asr	AAT Asr 910	TCT Ser	GAA Glu		2736
10	AAT Asn	AGT Ser	AAT Asn 915		ACG Thr	GCA Ala	CAT His	CAA Gln 920	Ser	AAA Lys	TTC Phe	GTT Val	GTA Val 925	. Tyr	GAT Asp	AGT Ser		2784
15	ATG Met	TTT Phe 930	- ASP	'AAT Asn	TTT Phe	AGC Ser	ATT Ile 935	AAC Asn	TTT Phe	TGG Trp	GTA Val	AGG Arg 940	Thr	CCT Pro	'AAA Lys	TAT		2832
20	945		7.51	nsp	116	CAA Gln 950	inr	ryr	Leu	GIn	Asn 955	Glu	Tyr	Thr	Ile	11e 960		2880
		-,-	110	Буз	965	GAC Asp	ser	GIY	Trp	1ys 970	Val	Ser	Ile	Lys	Gly 975	Asn		2928
25				980	1111	TTA Leu	iie	Asp	985	Asn	Ala	Lys	Ser	Lys 990	Ser	Ile		2976
30			995	. 7 .	261	ATA Ile	Lys	1000	Asn	ITE	Ser	Asp	Tyr 100	Ile 5	Asn	Lys		3024
35		1010) .	116	1111	ATT Ile	1015	Asn	Asp	Arg	Leu	Gly 1020	Asn)	Ala	Asn	Ile		3072
40	TAT Tyr 1025			Gry	361	1030	Бүз	ьуs	ser	GIU	Lys 1035	Ile	Leu	Asn	Leu	Asp 1040		3120
1.5	AGA . Arg		7011	361	1045	ASII	Asp	iie	Asp	Phe 1050	Lys	Leu	Ile	Asn	Cys 1055	Thr		3168
45	GAT A			1060	FILE	vai	irp	116	Lys 1065	Asp	Phe	Asn	Ile	Phe 1070	Gly	Arg		3216
50	GAA 1 Glu I		1075	AIG	1111	Giu	val :	ser 1080	Ser	Leu	Tyr	Trp	Ile 1085	Gln	Ser	Ser		3264
55		1090		Dea	Lys .	rsp :	1095	irp	GIA	Asn	Pro .	Leu 1100	Arg	Tyr	Asp	Thr		3312
60	CAA 1 Gln 1 1105		. y <u>.</u> .	beu	rne .	1110	oin (ı Are	Met	GIn 1	Asn . 1115	Ile	Tyr	Ile	Lys '	Tyr 1120		3360
	TTT A	GT / Ger I	AAA (nia .	TCT I Ser I 1125	ATG C	GG (GAA / Glu :	rhr A	GCA (Ala I 1130	CCA (Pro /	CGT . Arg	ACA I	Asn :	TTT A Phe A	AAT Asn	;	3408

,	AAT GCA GCA ATA AAT TAT CAA AAT TTA TAT CTT GGT TTA CGA TTT ATT Asn Ala Ala Ile Asn Tyr Gln Asn Leu Tyr Leu Gly Leu Arg Phe Ile 1140 1145 1150	3456
.5	ATA AAA AAA GCA TCA AAT TCT CGG AAT ATA AAT AAT GAT AAT ATA GTC Ile Lys Lys Ala Ser Asn Ser Arg Asn Ile Asn Asn Asp Asn Ile Val 1155 1160 1165 .	3504
10	AGA GAA GGA GAT TAT ATA TAT CTT AAT ATT GAT AAT ATT TCT GAT GAA Arg Glu Gly Asp Tyr Ile Tyr Leu Asn Ile Asp Asn Ile Ser Asp Glu 1170 1175 1180	3552
15	TCT TAC AGA GTA TAT GTT TTG GTG AAT TCT AAA GAA ATT CAA ACT CAA Ser Tyr Arg Val Tyr Val Leu Val Asn Ser Lys Glu Ile Gln Thr Gln 1185 1190 1195 1200	3600
20	TTA TTT TTA GCA CCC ATA AAT GAT GAT CCT ACG TTC TAT GAT GTA CTA Leu Phe Leu Ala Pro Ile Asn Asp Asp Pro Thr Phe Tyr Asp Val Leu 1205 1210	3648
	CAA ATA AAA AAA TAT TAT GAA AAA ACA ACA TAT AAT TGT CAG ATA CTT Gln Ile Lys Lys Tyr Tyr Glu Lys Thr Thr Tyr Asn Cys Gln Ile Leu 1220 1225 1230	3696
25	Cys Glu Lys Asp Thr Lys Thr Phe Gly Leu Phe Gly Ile Gly Lys Phe 1235 1240 1245	3744
30	GTT AAA GAT TAT GGA TAT GTT TGG GAT ACC TAT GAT AAT TAT TTT TGC Val Lys Asp Tyr Gly Tyr Val Trp Asp Thr Tyr Asp Asn Tyr Phe Cys 1250 1260	3792
35	ATA AGT CAG TGG TAT CTC AGA AGA ATA TCT GAA AAT ATA AAT AAA TTA Ile Ser Gln Trp Tyr Leu Arg Arg Ile Ser Glu Asn Ile Asn Lys Leu 1270 1275 1280	3840
40	AGG TTG GGA TGT AAT TGG CAA TTC ATT CCC GTG GAT GAA GGA TGG ACA Arg Leu Gly Cys Asn Trp Gln Phe Ile Pro Val Asp Glu Gly Trp Thr 1285 1290 1295	3888
	GAA TAA Glu	3894
45	(2) INFORMATION FOR SEQ ID NO:77: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 1297 amino acids	
50	(B) TYPE: amino acid (D) TOPOLOGY: linear (ii) MOLECULE TYPE: protein	
.	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:	
55	Met Pro Val Asn Ile Lys Asn Phe Asn Tyr Asn Asp Pro Ile Asn Asn 1 5 10 15	
60	Asp Asp Ile Ile Met Met Glu Pro Phe Asn Asp Pro Gly Pro Gly Thr 20 25 30	
	Tyr Tyr Lys Ala Phe Arg Ile Ile Asp Arg Ile Trp Ile Val Pro Glu 35 40 45	
65	Arg Phe Thr Tyr Gly Phe Gln Pro Asp Gln Phe Asn Ala Ser Thr Gly 50 55 60	
70	Val Phe Ser Lys Asp Val Tyr Glu Tyr Tyr Asp Pro Thr Tyr Leu Lys 65 70 75 80	
70	Thr Asp Ala Glu Lys Asp Lys Phe Leu Lys Thr Met Ile Lys Leu Phe	

1	•				85					90)				95	5
5	Asr	Arg	Ile	Asn 100	Ser	Lys	Pro	Ser	Gly 105	Gln	Arg	Leu	. Leu	Asp 110		: Ile
	Val	. Asp	Ala 115	Ile	Pro	Tyr	Leu	Gly 120	Asn	Ala	Ser	Thr	Pro 125		Asp	Lys
10	Phe	Ala 130	Ala	Asn	Val	Ala	Asn 135	Val	Ser	Ile	Asn	Lys 140		Ile	Ile	Gln
	Pro 145	Gly	Ala	Glu	Asp	Gln 150	Ile	Lys	Gly	Leu	Met 155		Asn	Leu	Lle	Ile 160
15	Phe	Gly	Pro	Gly	Pro 165	Val	Leu	Ser	Asp	Asn 170	Phe	Thr	Asp	Ser	Met 175	Ile
20	Met	Asn	Gly	His 180	Ser	Pro	Ile	Ser	Glu 185	Gly	Phe	Gly	Ala	Arg 190	Met	Met
	Ile	Arg	Phe 195	Cys	Pro	Ser	Cys	Leu 200	Asn	Val	Phe	Asn	Asn 205	Val	Gln	Glu
25	Asn	Lys 210	Asp	Thr	Ser	Ile	Phe 215	Ser	Arg	Arg	Ala	Tyr 220	Phe	Ala	Asp	Pro
	Ala 225	Leu	Thr	Leu	Met	His 230	Glu	Leu	Ile	His	Val 235	Leu	His	Gly	Leu	Tyr 240
30	Gly	Ile	Lys	Ile	Ser 245	Asn	Leu	Pro	Ile	Thr 250	Pro	Asn	Thr	Lys	Glu 255	Phe
35	Phe	Met	Gln	His 260	Ser	Asp	Pro	Val	Gln 265	Ala	Glu	Glu	Leu	Tyr 270	Thr	Phe
	Gly	Gly	His 275	Asp	Pro	Ser	Val	Ile 280	Ser	Pro	Ser	Thr	Asp 285	Met	Asn	Ile
40	Tyr	Asn 290	Lys	Ala	Leu	Gln	Asn 295	Phe	Gln	Asp	Ile	Ala 300	Asn	Arg	Leu	Asn
	Ile 305	Val	Ser	Ser	Ala	Gln 310	Gly	Ser	Gly	Ile	Asp 315	Ile	Ser	Leu	Туr	Lys 320
45	Gln	Ile	Tyr	Lys	Asn 325	Lys	Tyr	Asp	Phe	Val 330	Glu	Asp	Pro	Asn	Gly 335	Lys
50	Tyr	Ser	Val	Asp 340	Lys	qzA	Lys	Phe	Asp 345	Lys	Leu	Tyr	Lys	Ala 350	Leu	Met
	Phe	Gly	Phe 355	Thr	Glu	Thr	Asn	Leu 360	Ala	Gly	Glu	Tyr	Gly 365	Ile	Lys	Thr
55	Arg	Tyr 370	Ser	Tyr	Phe	Ser	Glu 375	Tyr	Leu	Pro	Pro	Ile 380	Lys	Thr	Glu	Lys
	Leu 385	Leu	Asp	Asn	Thr	Ile 390	Tyr	Thr	Gln	Asn	Glu 395	Gly	Phe	Asn	Ile	Ala 400
60	Ser	Lys	Asn	Leu	Lys 405	Thr	Glu	Phe	Asn	Gly 410	Gln	Asn	Lys	Ala	Val 415	Asn
65	Lys	Glu	Ala	Tyr 420	Glu	Glu	Ile	Ser	Leu 425	Glu	His	Leu	Val	Ile 430	Tyr	Λrg
-	Ile	Ala	Met 435	Суѕ	Lys	Pro	Val	Met 440	Tyr	Lys	Asn	Thr	Gly 445	Lys	Ser	Glu
70	Gln	Cys 450	Ile	Ile	Val	Asn	Asn 455	Glu	Asp	Leu	Phe	Phe 460	Ile	Ala	Asn	Lys

70

	465	Ser	Phe	Ser	Lys	470	Leu	Ala	Lys	Ala	475	Thr	Ile	Ala	Туг	Asn 480
5	Thr	Gln	Asn	Asn	Thr 485	Ile	Glu	Asn	Asn	Phe 490	s Ser	Ile	Asp	Gln	Leu 495	Ile
	Leu	Asp	Asn	Asp 500	Leu	Ser	Ser	Gly	1le 505	Asp	Leu	Pro	Asn	Glu 510	Asn	Thr
10	Glu	Pro	Phe 515	Thr	Asn	Phe	Asp	Asp 520	Ile	Asp	Ile	Pro	Val 525	Туг	Ile	Lys
15							235					540				Glu
						330					555					560
20	Thr	Asn	Ser	Leu	Asn 565	Asp	Ala	Leu	Arg	Asn 570	Asn	Asn	Lys	Val	Tyr 575	Thr
•		Phe		300					585					590		
25		Leu	,,,,					600					605			
30		Ser 610					013					620				
		Ile				630					635					640
35		Glu			043					650					655	
40		Glu		000					665					670		
40		Glu						000					685			
45		Ala 690					075					700				
	. 0 3	Val				/10					715					720
50		Glu			123					730					735	
55		Ile		740					745					750		
).1		Ile	, , ,					760					765			
50		770					//5					780				
	, 0 5	Ser				790					795					800
5		Asp			005					810					815	
'O		Asn		020					825					830		
V	val	Asn	Arg	His	Leu	Lys	Asp	Ser	Ile	Pro	Phe	Asp	Leu	Ser	Leu	Tyr

			835					840					845			
5	Thr	Lys 850	qaA	Thr	Ile	Leu	Ile 855	Gln	Val	Phe	Asn	Asn 860	Tyr	Ile	Ser	Asn
•	Ile 865	Ser	Ser	Asn	Ala	Ile 870	Leu	Ser	Leu	Ser	Tyr 875	Arg	Gly	Gly	Arg	Leu 880
10	Ile	Asp	Ser	Ser	Gly 885	Tyr	Gly	Ala	Thr	Met 890		Val	Gly	Ser	Asp 895	Val
	Ile	Phe	Asn	Asp 900	Ile	Gly	Asn	Gly	Gln 905	Phe	Lys	Leu	Asn	Asn 910	Ser	Glu
15	Asn	Ser	Asn 915	Ile	Thr	Ala	His	Gln 920	Ser	Lys	Phe	Val	Val 925	Tyr	Asp'	Ser
20	Met	Phe 930	qzA	Asn	Phe	Ser	Ile 935	Asn	Phe	Trp	Val	Arg 940	Thr	Pro	Lys	Tyr
	Asn 945	Asn	Asn	Asp	Ile	Gln 950	Thr	Tyr	Leu	Gln	Asn 955	Glu	туг	Thr	Ile	11e 960
25					965		Ser			970					975	
•				980			Ile		985					990		
30			995				Lys	1000)				1009	5		
35		1010					Thr 1019	5				1020)			
	1025	5				1030					1035	5				1040
40					1045	5	Asp			1050	3				1055	5
1.5				1060)		Trp		1065	5				1070)	
45			1075	5			Val	1080)				1085	5		
50		1090)				Phe 1099	•				1100)			
	1109	5				1110					1115	5				1120
55					1125	5	Gly			1130)				1135	5
60				1140)		Gln		1145	5				1150)	
60			1155	5			Ser	1160)				1165	5		
65		1170)				Tyr 1175	•				1180)			
	1185	•				1190					1195	i				1200
70	Leu	Phe	Leu	Ala	Pro 1205	Ile	Asn	Asp	Asp	Pro		Phe	Tyr	Asp	Val	

	Gln Ile Lys Lys Tyr Tyr Glu Lys Thr Thr Tyr Asn Cys Gln Ile Leu 1220 1225 1230	
5	Cys Glu Lys Asp Thr Lys Thr Phe Gly Leu Phe Gly Ile Gly Lys Phe 1235 1240 1245	
•	Val Lys Asp Tyr Gly Tyr Val Trp Asp Thr Tyr Asp Asn Tyr Phe Cys 1250 1255 1260	
10	Ile Ser Gln Trp Tyr Leu Arg Arg Ile Ser Glu Asn Ile Asn Lys Leu 1265 1270 1275 1280	
15	Arg Leu Gly Cys Asn Trp Gln Phe Ile Pro Val Asp Glu Gly Trp Thr 1285 1290 1295	
	Glu	
	(2) INFORMATION FOR SEQ ID NO:78:	
20	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 1535 base pairs (B) TYPE: nucleic acid	
25	(C) STRANDEDNESS: double (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic)	
30	(ix) FEATURE: (A) NAME/KEY: CDS (B) LOCATION: 1081526	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:	
35	AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA	6
	TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACC ATG GGC CAT	
	Met Gly His	110
40	CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT His His His His His His His Ser Ser Gly His Ile Glu Gly 5	164
45	CGT CAT ATG GCT AGC ATG GCT GAC ACA ATT TTA ATA CAA GTT TTT AAT Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Gln Val Phe Asn 20 25 30 35	212
50	AAT TAT AGT AAT ATT AGT AGT AAT GCT ATT TTA AGT TTA AGT TAT ASN Tyr Ile Ser Asn Ile Ser Ser Asn Ala Ile Leu Ser Leu Ser Tyr 40 45 50	260
55	AGA GGT GGG CGT TTA ATA GAT TCA TCT GGA TAT GGT GCA ACT ATG AAT Arg Gly Gly Arg Leu Ile Asp Ser Ser Gly Tyr Gly Ala Thr Met Asn 55 60 65	308
	GTA GGT TCA GAT GTT ATC TTT AAT GAT ATA GGA AAT GGT CAA TTT AAA Val Gly Ser Asp Val Ile Phe Asn Asp Ile Gly Asn Gly Gln Phe Lys 70 75 80	356
60	TTA AAT AAT TCT GAA AAT AGT AAT ATT ACG GCA CAT CAA AGT AAA TTC Leu Asn Asn Ser Glu Asn Ser Asn Ile Thr Ala His Gln Ser Lys Phe 85 90 95	404
65	GTT GTA TAT GAT AGT ATG TTT GAT AAT TTT AGC ATT AAC TTT TGG GTA Val Val Tyr Asp Ser Met Phe Asp Asn Phe Ser Ile Asn Phe Trp Val 100 115	452
70	AGG ACT CCT AAA TAT AAT AAT AAT GAT ATA CAA ACT TAT CTT CAA AAT Arg Thr Pro Lys Tyr Asn Asn Asn Asp Ile Gln Thr Tyr Leu Gln Asn 120 125 130	500

	GA(Glu	TA'	T ACZ	A ATA	5 116	r AGT e Ser	TGT Cys	T ATA	A AAA B Lys 140	Ası	r GAG 1 Asp	TC/ Sei	A GG/	A TGO	Ly:	A GTA s Val	548
5	TCT Sei	T ATT	r AAC ≥ Lys	, GT	A AA7 ⁄ Asr	F AGA n Arg	ATA	A ATA	rr	ACA Thi	TTA	A ATA	GAT ASP 160	va)	C AA?	r GCA n Ala	596
10	AAA Lys	TC1 S Se1 165	. Lys	TC! Ser	ATA Ile	A TTT	TTC Phe 170	GIU	A TAT	AGT Ser	ATA	1 AAA 179	Asp	TAA T	T ATA	A TCA Ser	644
15	GAT Asp 180	, 1 y 1	T ATA	AAT Asn	`AAA Lys	TGG Trp 185	Pne	TCC Ser	TATA	ACT Thr	ATT Ile 190	Thr	`AAT Asn	GAI Asp	AGA	TTA Leu 195	692
20	Giy	ASI.	HIA	ASI	200	Tyr	11e	Asn	Gly	Ser 205	Leu	Lys	Lys	Ser	Glu 210		740
	ATT Ile	TTA Leu	AAC Asn	TTA Leu 215	Asp	AGA Arg	ATT Ile	AAT Asn	TCT Ser 220	Ser	AAT Asn	GAT Asp	ATA Ile	GAC Asp 225	Phe	AAA Lys	788
25	beu	116	230	Cys	Thr	Asp	Thr	Thr 235	Lys	Phe	Val	Trp	11e 240	Lys	Asp		836
30	AAT Asn	ATT Ile 245	FIIE	GGT Gly	AGA Arg	GAA Glu	TTA Leu 250	AAT Asn	GCT Ala	ACA Thr	GAA Glu	GTA Val 255	TCT Ser	TCA Ser	CTA Leu	TAT Tyr	884
35	260	116	GIN	ser	ser	265	Asn	Thr	Leu	Lys	Asp 270	Phe	Trp	Gly	Asn	2 75	932
40	TTA Leu	AGA Arg	TAC Tyr	GAT Asp	ACA Thr 280	CAA Gln	TAC Tyr	TAT Tyr	CTG Leu	TTT Phe 285	AAT Asn	CAA Gln	GGT Gly	ATG Met	CAA Gln 290	AAT Asn	980
	ATC Ile	TAT Tyr	ATA Ile	AAG Lys 295	TAT Tyr	TTT Phe	AGT Ser	AAA Lys	GCT Ala 300	TCT Ser	ATG Met	GGG Gly	GAA Glu	ACT Thr 305	GCA Ala	CCA Pro	1028
45	Arg	1111	310	Pne	ASI	AAT Asn	Ala	315	Ile	Asn	Tyr	Gln	Asn 320	Leu	Tyr	Leu	1076
50	GGT Gly	TTA Leu 325	CGA Arg	TTT Phe	ATT Ile	ATA Ile	AAA Lys 330	AAA Lys	GCA Ala	TCA Ser	AAT Asn	TCT Ser 335	CGG Arg	AAT Asn	ATA Ile	AAT Asn	1124
55	340	vab	ASII	116	vai	AGA Arg 345	GIU	GIY	Asp	Tyr	11e 350	Tyr	Leu	Asn	Ile	Asp 355	1172
60	ASII	116	ser	Asp	360	TCT Ser	Tyr	Arg	Val	Tyr 365	Val	Leu	Val	Asn	Ser 370	Lys	1220
	GAA Glu	ATT Ile	CAA Gln	ACT Thr 375	CAA Gln	TTA Leu	TTT Phe	TTA Leu	GCA Ala 380	CCC Pro	ATA Ile	TAA Asn	GAT Asp	GAT Asp 385	CCT Pro	ACG Thr	1268
65	TTC Phe	TAT Tyr	GAT Asp 390	GTA Val	CTA Leu	CAA Gln	TIE	AAA Lys 395	AAA Lys	TAT Tyr	TAT Tyr	GAA Glu	AAA Lys 400	ACA Thr	ACA Thr	TAT Tyr	1316
70	AAT Asn	TGT Cys	CAG Gln	ATA Ile	CT T Leu	TGC Cys	GAA Glu	AAA Lys	GAT Asp	ACT Thr	AAA Lys	ACA Thr	TTT Phe	GGG Gly	CTG Leu	TTT Phe	1364

		405					410					415					•
	CCA											415					
5	Gly 420	116	Gly	Lys	Phe	Val 425	AAA Lys	GAT Asp	TAT	GGA Gly	TAT Tyr 430	GTT Val	TGG Trp	GAT Asp	ACC Thr	TAT Tyr 435	1412
10	GAT Asp	AAT Asn	TAT	TTT Phe	TGC Cys 440	ATA Ile	AGT Ser	CAG Gln	TGG Trp	TAT Tyr 445	CTC Leu	AGA Arg	AGA Arg	ATA Ile	TCT Ser 450	GAA Glu	1460
10	TAA Asn	ATA Ile	AAT Asn	AAA Lys 455	TTA Leu	AGG Arg	TTG Leu	GGA Gly	TGT Cys 460	AAT Asn	TGG Trp	CAA Gln	TTC Phe	ATT Ile	CCC Pro	GTG Val	1508
15	GAT Asp	GAA Glu	GGA Gly 470	TGG Trp	ACA Thr	GAA Glu	TAA	CTCG.	AG								1535
20	(2)	INF	ORMA	TION	FOR	SEQ	ID 1	NO : 7	9 :								
35			(i) .	(B)) LEI) TYI	NGTH PE: 8	RACTI : 471 amino GY: 1	am:	ino a id	: acids	5						
25		(:	ii)	MOLE													
		(:	xi) :	SEQUI	ENCE	DESC	CRIPT	пои	: SEC	OID	NO: 7	79:					
30	Met 1	Gly	His	His	His 5	His	His	His	His	His 10	His	His	Ser	Ser	Gly 15	His	
35	Ile	Glu	Gly	Arg 20	His	Met	Ala	Ser	Met 25	Ala	Asp	Thr	Ile	Leu 30	Ile	Gln	
	Val	Phe '	Asn 35	Asn	Tyr	Ile	Ser	Asn 40	Įle	Ser	Ser	Asn	Ala 45	Ile	Leu	Ser	
40	Leu	Ser 50	Tyr	Arg	Gly	Gly	Arg 55	Leu	Ile	Asp	Ser	Ser 60	Gly	Tyr	Gly	Ala	
	Thr 65	Met	Asn	Val	Gly	Ser 70	Asp	Val	Ile	Phe	Asn 75	Asp	Ile	Gly	Asn	Gly 80	
45	Gln	Phe	Lys	Leu	Asn 85	Asn	Ser	Glu	Asn	Ser 90	Asn	Ile	Thr	Ala	His 95	Gln	
50				Val 100					105					110			
			115	Arg				120					125			-	
55		130		Glu			135					140					
	145			Ser		150					155					160	
60				Lys	165					170					175		
65				Asp 180					185					190			
			195	Gly				200					205				
70	Ser	Glu 210	Lys	Ile	Leu	Asn	Leu 215	Asp	Arg	Ile	Asn	Ser 220	Ser	Asn	Asp	Ile	

	Asp 225	Phe	Lys	Leu	Ile	Asn 230	Cys	Thr	Asp	Thr	Thr 235	Lys	Phe	Val	Trp	11e 240	
5	Lys	Asp	Phe	Asn	Ile 245	Phe	Gly	Arg	Glu	Leu 250	Asn	Ala	Thr	Glu	Val 255	Ser	
	Ser	Leu	Tyr	Trp 260	Ile	Gln	Ser	Ser	Thr 265	Asn	Thr	Leu	Lys	'Asp 270		Trp	
10	Gly	Asn	Pro 275	Leu	Arg	Tyr	Asp	Thr 280	Gln	Tyr	Tyr	Leu	Phe 285	Asn	Gln	Gly	
15	Met	Gln 290	Asn	Ile	Tyr	Ile	Lys 295	Tyr	Phe	Ser	Lys	Ala 300	Ser	Met	Glγ	Glu	
	Thr 305	Ala	Pro	Arg	Thr	Asn 310	Phe	Asn	Asn	Ala	Ala 315	Ile	Asn	Tyr	Gln	Asn 320	
20	Leu	Tyr	Leu	Gly	Leu 325	Arg	Phe	Ile	Ile	Lys 330	Lys	Ala	Ser	Asn	Ser 335	Arg	
	Asn	Ile	Asn	Asn 340	Asp	Asn	Ile	Val	Arg 345	Glu	Gly	Asp	Tyr	Ile 350	Tyr	Leu	
25	Asn	Ile	Asp 355	Asn	Ile	Ser	Asp	Glu 360	Ser	Tyr	Arg	Val	Tyr 365	Val	Leu	Val	
30	Asn	Ser 370	Lys	Glu	Ile	Gln	Thr 375	Gln	Leu	Phe	Leu	Ala 380	Pro	Ile	Asn	Asp	
.,()	Asp 385	Pro	Thr	Phe	Tyr	Asp 390	Val	Leu	Gln	Ile	Lys 395	Lys	Tyr	Tyr	Glu	Lys 400	
35	Thr	Thr	Tyr	Asn	Cys 405	Gln	Ile	Leu	Cys	Glu 410	Lys	Asp	Thr	Lys	Thr 415	Phe	
	Gly	Leu	Phe	Gly 420	Ile	Gly	Lys	Phe	Val 425	Lys	Asp	Tyr	Gly	Tyr 430	Val	Trp	
40	Asp	Thr	Tyr 435	Asp	Asn	Tyr	Phe	Cys 440	Ile	Ser	Gln	Trp	Tyr 445	Leu	Arg	Arg	
45	Ile	Ser 450	Glu	Asn	Ile	Asn	Lys 455	Leu	Arg	Leu	Gly	Cys 460	Asn	Trp	Gln	Phe	
7.1	11e 465	Pro	Val	Asp	Glu	Gly 470	Trp	Thr	Glu								
=0	(2)	INFO	RMAT	CION	FOR	SEQ	ID N	10:80) :								
50		(i)	(<u>P</u>	A) LE B) TY	NGTH	IARAC I: 30 nucl	bas eic	se pa	irs								
55						EDNE GY:			jle								
•		(ii)							clei								
60		(xi)	SEC	UENC	E DE	SCRI	PTIC	ON: S	SEQ I	D NO	:80:						
	CGCC	ATGG	CT G	ACAC	CAATI	T TA	ATAC	CAAGT	7								3 (
65	(2)	INFO	RMAT	NOI	FOR	SEQ	ID N	10:81	:								
		(i)	A)) LE	NGTH	ARAC I: 32	bas	se pa	irs								
70			(C) ST	'RANE	EDNE	SS:	sing									

- 379 -

	<pre>(11) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA"</pre>	
5	(x1) SEQUENCE DESCRIPTION: SEQ ID NO:81:	
-	GCCTCGAGTT ATTCTGTCCA TCCTTCATCC AC	-
	(2) INFORMATION FOR SEQ ID NO:82:	-
10	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 12 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: not relevant	
15	(D) TOPOLOGY: not relevant	
	(ii) MOLECULE TYPE: peptide	
20	(ix) FEATURE: (A) NAME/KEY: Modified-site (B) LOCATION: 12 (D) OTHER INFORMATION: (a.e., p. 1)	
	(D) OTHER INFORMATION: /note= "The asparagine residue at this position contains an amide group."	
25	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:	
	Cys Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn	

5

15

30

CLAIMS

- 1. A host cell containing a recombinant expression vector, said vector encoding a protein comprising at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin.
- 2. The host cell of Claim 1, wherein and said host cell is capable of expressing said protein at a level greater than or equal to 5% of the total cellular protein.
- 3. The host cell of Claim 1, wherein and said host cell is capable of expressing said protein as a soluble protein at a level greater than or equal to 0.25% of the total soluble cellular protein.
 - 4. The host cell of Claim 1, wherein said host cell is an Escherichia coli cell.
 - 5. The host cell of Claim 1, wherein said host cell is an insect cell.
 - .6. The host cell of Claim 1, wherein said host cell is a yeast cell.
- 7. A host cell containing a recombinant expression vector, said vector encoding a fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin.
- 25 8. The host cell of Claim 7, wherein said portion of said toxin comprises the receptor binding domain.
 - 9. The host cell of Claim 7, wherein said non-toxin protein sequence comprises a poly-histidine tract.
 - 10. A vaccine comprising a fusion protein, said fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium hotulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin.

11. The vaccine of Claim 10 further comprising a fusion protein comprising a non-toxin protein sequence and at least a portion of *Clostridium hotulinum* type A toxin.

- 12. The vaccine of Claim 10, wherein said portion of said Clostridium botulinum toxin comprises the receptor binding domain.
- 13. The vaccine of Claim 10 wherein said non-toxin protein sequence comprises a poly-histidine tract.
 - 14. The vaccine of Claim 10, wherein said vaccine is substantially endotoxin-free.
- 15. A method of generating antibody directed against a Clostridium botulinum toxin comprising:
 - a) providing in any order:

5

10

15

20

25

30

- i) an antigen comprising a fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin. said toxin selected from the group consisting of type B toxin and type E toxin, and
 - ii) a host; and
- b) immunizing said host with said antigen so as to generate an antibody.
- 16. The method of Claim 15, wherein said antigen further comprises a fusion protein comprising a non-toxin protein sequence and at least a portion of *Clostridium hotulinum* type A toxin.
- 17. The method of Claim 15, wherein said portion of said *Clostridium botulinum* toxin comprises the receptor binding domain.
- 18. The method of Claim 15 wherein said non-toxin protein sequence comprises a poly-histidine tract.
 - 19. The method of Claim 15 wherein said host is a mammal.
 - 20. The method of Claim 19 wherein said mammal is a human.

21. The method of Claim 15 further comprising step c) collecting said antibodies from said host.

- 22. The method of Claim 21 further comprising step d) purifying said antibodies.
- 23. The antibody raised according to the method of Claim 15.
- 24. The antibody raised according to the method of Claim 16.

- 383 -

FIGURE 1

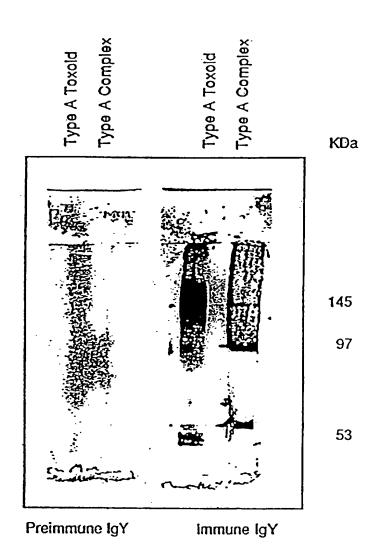
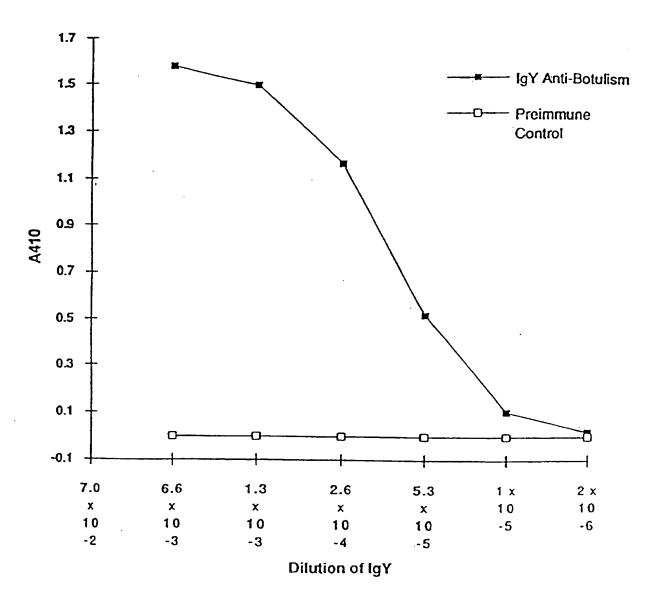


FIGURE 2



2/40

FIGURE 3

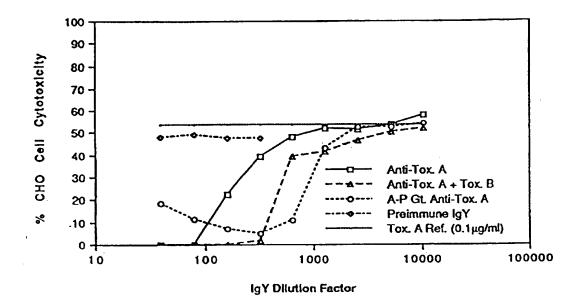


FIGURE 4

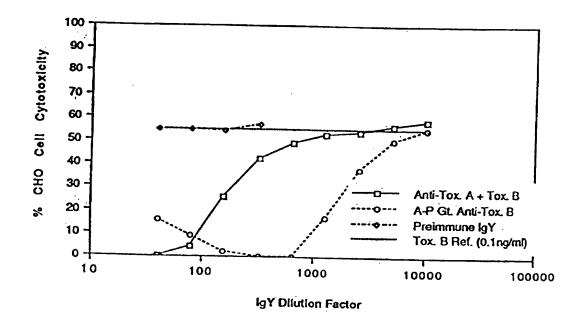


FIGURE 5

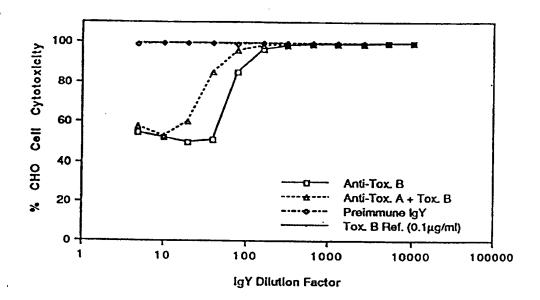
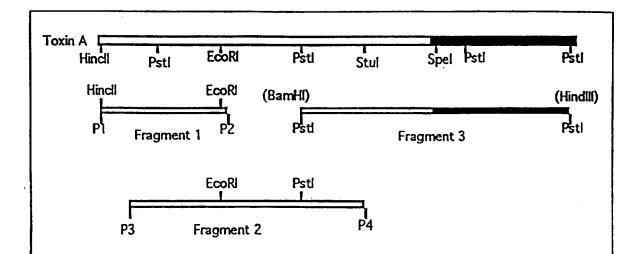


FIGURE 6



P1-P4 are PCR primers 1-4. P1=5'GGAAATTTAGCTGCAGCATCTGAC3', P2=5'TCTAGCAAATTCGCTTGTGTTGAA3',P3=5'CTCGCATATAGCATTAGACC3', P4=5'CTATCTAGGCCTAAAGTAT3'. Indicated restriction sites in fragments 1 and 2 are internal sites used to done into pGEX2T vector (fragment 1; construct called pGA30-660) or pMALc vector (fragment 2; construct called pMA660-1100). Bracketed restriction sites at ends of fragment 3 are pUC9 polylinker sites utilized to clone fragment 3 into pET23 vector (construct called pPA1100-2680). Numbers in these constructs refer to toxin A amino acid interval that is expressed. The shaded portion of the toxin A gene corresponds to the repeating ligand binding domain.

FIGURE 7

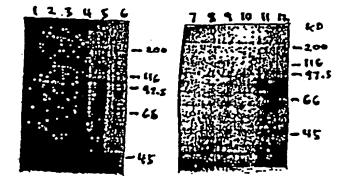


FIGURE 8

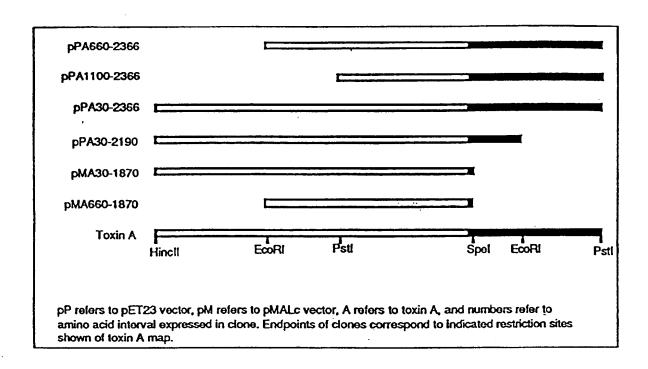


FIGURE 9

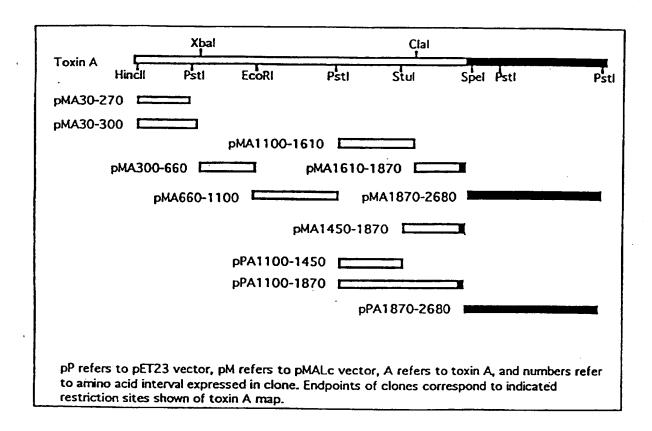


FIGURE 10

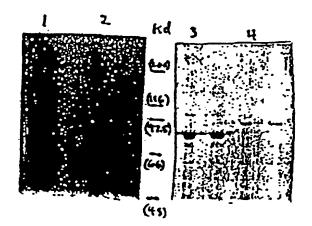


FIGURE 11

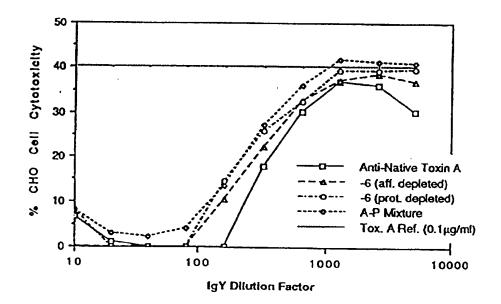
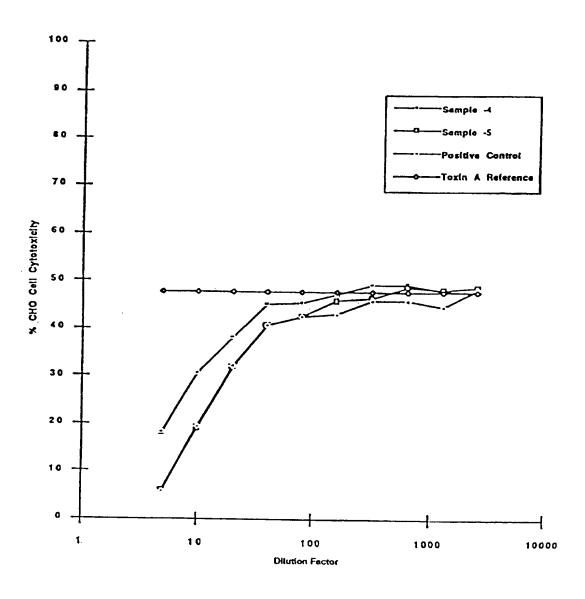


FIGURE 12



12/40

FIGURE 13

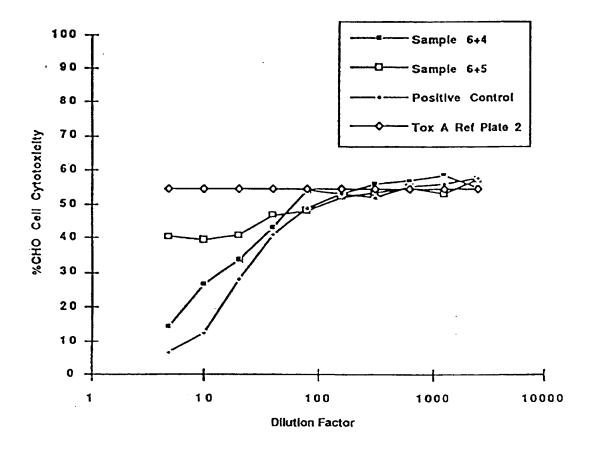


FIGURE 14

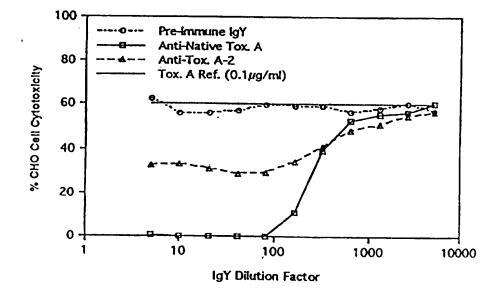
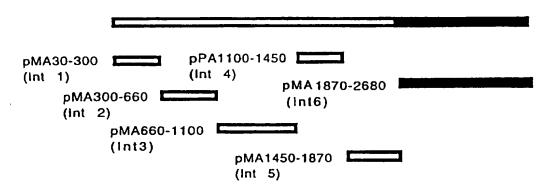


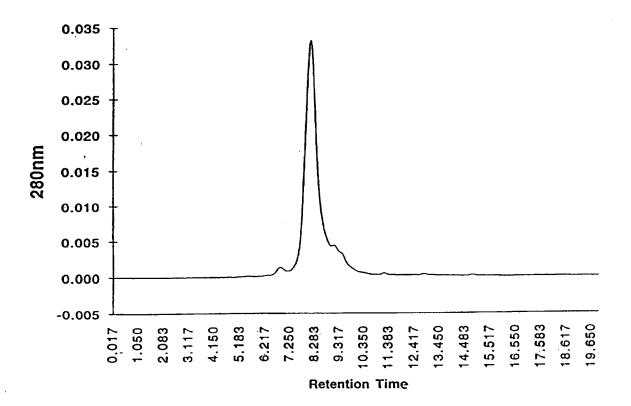
FIGURE 15

A.

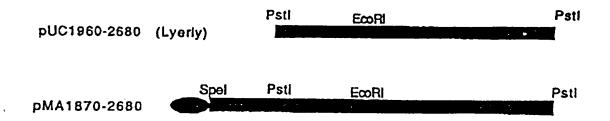


В Pstl Spel EcoRI Soluble pMA1870-2680 E∞RI Insoluble pPA1870-2680(H) HHH Insoluble pPA1870-2680 Insoluble pBA1870-2680 Insoluble pGA1870-2190 Insoluble pBA1870-2190 insoluble pPA1870-2190 HH insoluble pMA1870-2190 Unstable pMA2250-2680 Insolubie pBA2250-2680

FIGURE 16



WO 98/08540



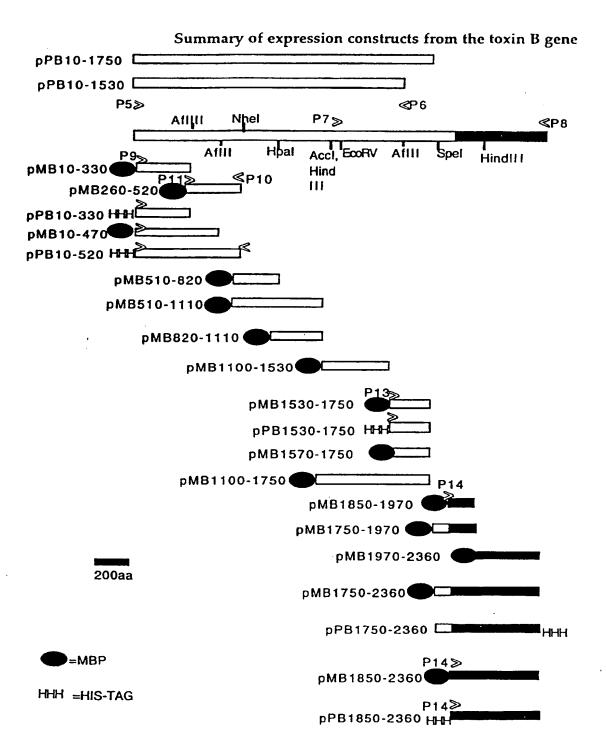
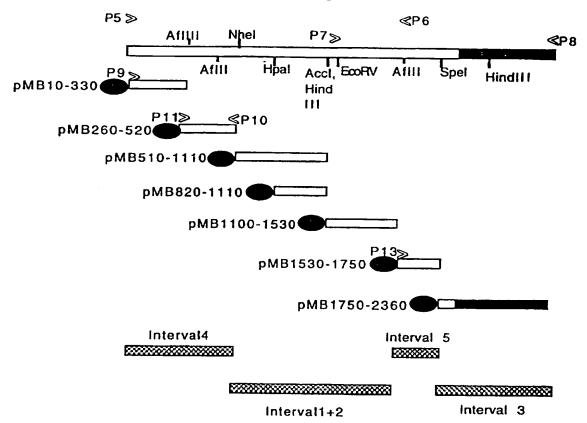


FIGURE 19

Interval specific expression constructs



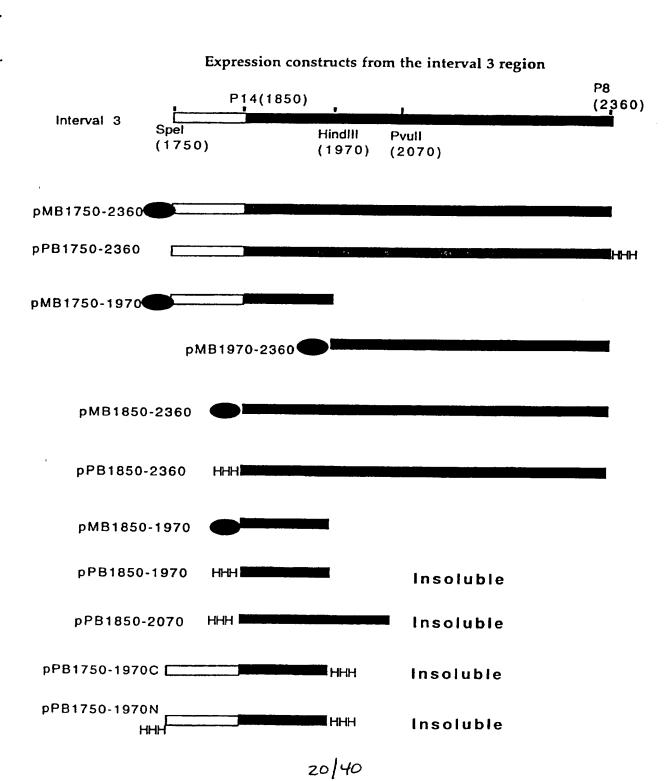
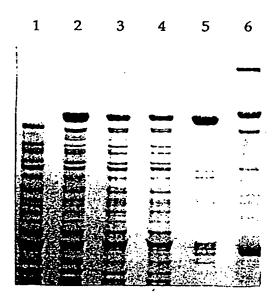


FIGURE 21



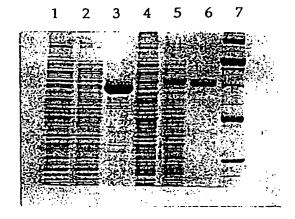
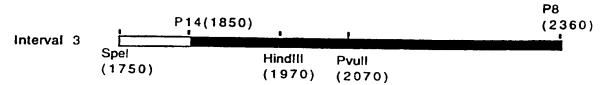


FIGURE 23

Binding of neutralizing CTB antibodies by recombinant toxin B protein



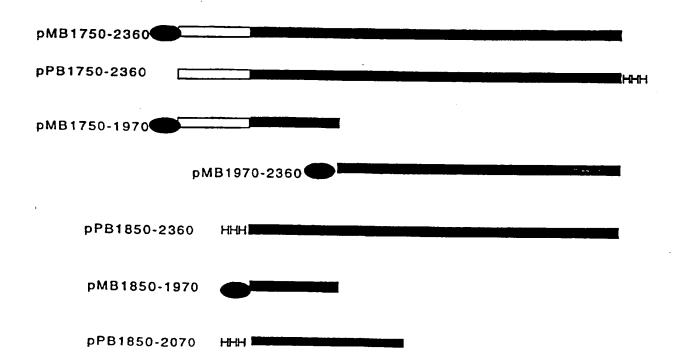
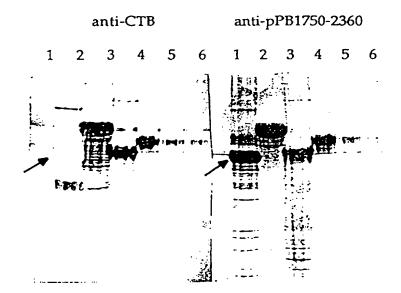
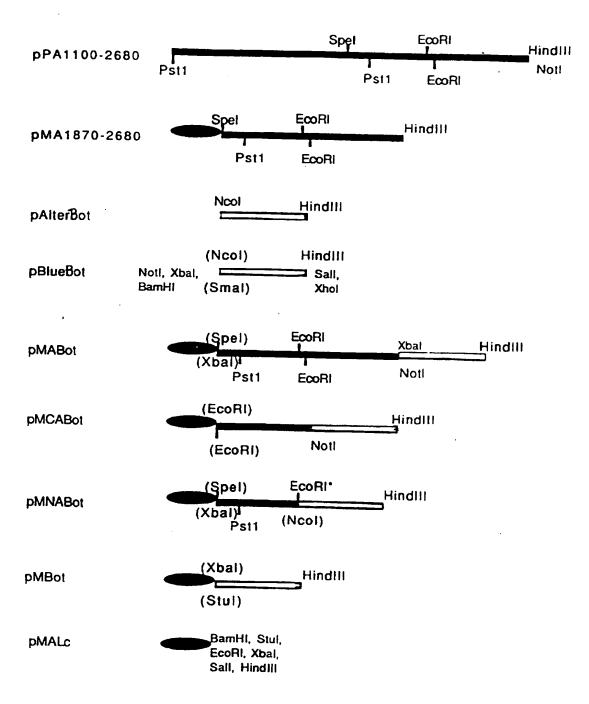
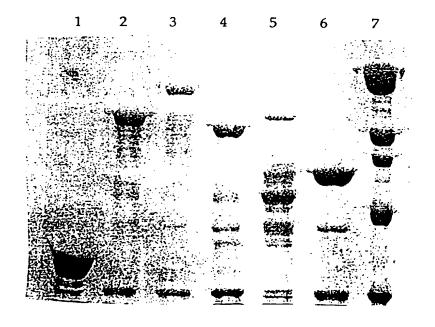


FIGURE 24







pAiterBot	N∞l	HindIII
	(Ncol)	HindIII
pBlueBot	Notl, Xbal, BamHl (Smal)	Sall, Xhol
pMBot	(Xbai)	Hindll
•	(Stul)	
pHisBot	(Ncol)	HindIII
	Ndel*	
pPBot	(Ncol)	HindIII
	(Notl)	(Sali)
pGBot	(Smål)	(XhoI)

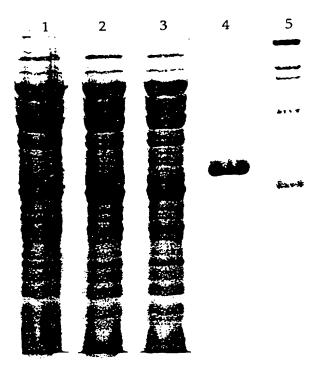
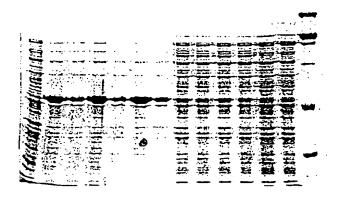
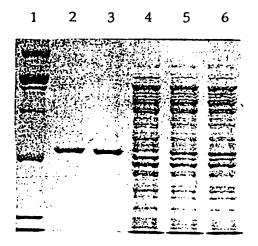
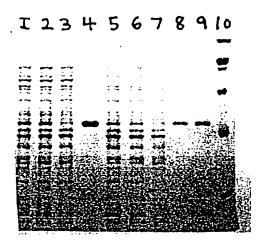


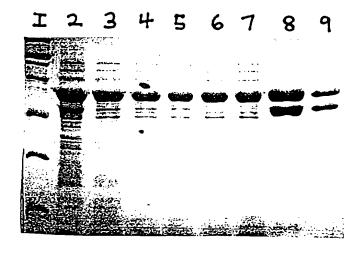
FIGURE 29

1 2 3 4 5 6 7 8 9 10 11 12 13 14

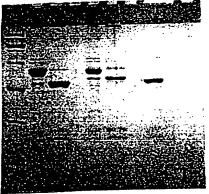


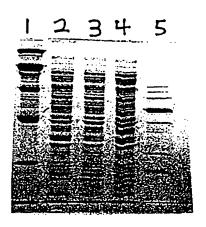












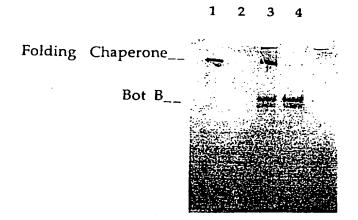


FIGURE 36

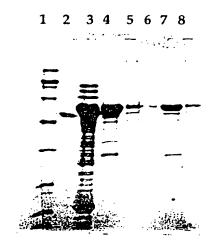


FIGURE 37

1 2 3 4 5

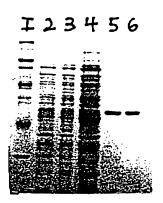
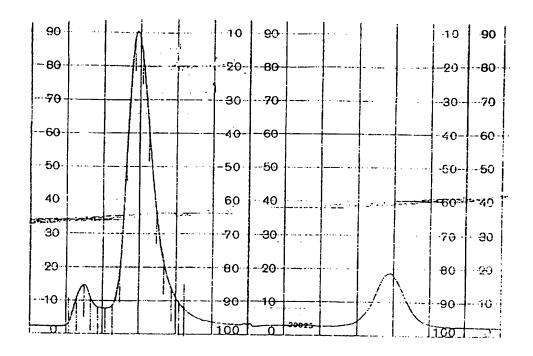




FIGURE 40



INTERNATIONAL SEARCH REPORT

International application No. PCT/US97/15394

A. CLASSIFICATION OF SUBJECT MATTER IPC(6) :Please See Extra Sheet.			
US CL :Please See Extra Sheet.			
According to International Patent Classification (IPC) or to bot	h national classification and IPC		
B. FIELDS SEARCHED	1.1.1		
Minimum documentation searched (classification system follow	•		
U.S. : 424/184.1,192.1, 247.1; 435/69.1, , 69.7, 325, 320.1	1; 530/388.4, 389.5		
Documentation searched other than minimum documentation to t	the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (MEDLINE, BIOSIS, WPIDS, CAPLUS, APS	name of data base and, where practicable, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT			
Category* Citation of document, with indication, where	appropriate, of the relevant passages Relevant to claim No.		
Y THOMPSON et al. The Complete Clostridium botulinum Type A Neuro Sequence Analysis of the Encoding C 1990, Vol. 189, pages 73-81, see en	otoxin, Deduced by Nucleotide Gene. Eur. J. Biochem. April		
Y BINZ et al. The Complete Sequence A and Comparison with Other Closts Biological Chemistry. June 1990, V 9158, see entire document.	ridial Neurotoxins. Journal of		
Y ROITT. Essential Immunology. O Publications. 1988, especially pages	· · · · · · · · · · · · · · · · · · ·		
X Further documents are listed in the continuation of Box	C. See patent family annex.		
Special categories of cated documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand		
"A" document defining the general state of the art which is not considered to be of particular relevance	the principle or theory underlying the invention		
R earlier document published on or after the international filing date	"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step		
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other	when the document is taken alone		
special reason (se specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is		
"O" document referring to an oral disclosure, use, exhibition or other means	combined with one or more other such documents, such combination being obvious to a person skilled in the art		
P document published prior to the international filing date but later than the priority date claimed	*&* document member of the same patent family		
Date of the actual completion of the international search	Date of mailing of the international search report		
10 OCTOBER 1997	2 3 DEC 1997		
Name and mailing address of the ISA/US			
Commissioner of Patents and Trademarks Box PCT	EVELYN RABIN		
Washington, D.C. 20231 Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196		
	and the second control of the second control		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15394

	FC1/039//133	74
C (Continue	stion). DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	SIEGEL. Human Immune Response to Botulinum Pentavalent (ABCDE) Toxoid Determined by a Neutralization Test and by an Enzyme-Linked Immunosorbent Assay. Journal of Clinical Microbiology. November 1988, Vol. 26, pages 2351-2356, see entire document.	1-24
Y	FORD et al. Fusion Tails for the Recovery and Purification of Recombinant Proteins. Protein Expression Purification. 1991, Vol. 2, pages 95-107, see entire document.	1-24
Y	LECLERC et al. Induction of Virus-Neutralizing Antibodies by Bacteria Expressing the C3 Poliovirus Epitope in the Periplasm. Journal of Immunology. April 1990, Vol. 144, pages 3174-3182, see entire document.	1-24
Y	KLEID. Using Genetically Engineered Bacteria for Vaccine Production. Annals New York Acad. Sci. 1983, Vol. 483, pages 23-30, see entire document.	1-24
,		

INTERNATIONAL SEARCH REPORT

International application No. PCT/US97/15394

A61K 39/00, 39/38, 38/08; C12P 21/06, 21/04, 21/08; C12N 15/00, 15/09, 15/63, 15/70, 15/74; C07K 16/00 A. CLASSIFICATION OF SUBJECT MATTER: US CL :			
•			
•			